EXHIBIT F

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TRANSFORMING GROWTH FACTOR-ALPHA INDUCES NEUROGENESIS AND BEHAVIORAL IMPROVEMENT IN A CHRONIC STROKE MODEL

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Abstract—Transforming growth factor-alpha (TGFα) is a powerful endogenous mitogen and neurotrophic factor, which has previously been shown to induce a massive proliferative response in the brains of Parkinson's disease model rats injured by an acute neurotoxic lesion. We now show that TGFα can also produce a massive proliferative response in rat brains subjected to stroke caused by a middle cerebral artery occlusion (MCAO), even when the growth factor is administered as late as 4 weeks after injury. This combination of stimuli provokes DNA synthesis, shown by 5′-bromo-2-deoxyuridine incorporation, throughout the ependymal layer and subventricular zone (SVZ) of the forebrain during the 4 weeks of growth factor administration. The newly generated cells migrate preferentially along and ventral to the corpus callosum (CC) and external capsule to the site of the injury where many of them differentiate into several site-appropriate neuronal phenotypes in association with near complete (99%) behavioral recovery. We conclude that the injury response of endogenous neural stem cells as well as behavioral recovery can be significantly enhanced by application of TGFα, and that this approach represents a potential therapeutic strategy for chronic stroke and other neurological damage in human patients.

Key words: adult stem cell, subventricular zone, injury, proliferation, differentiation, neurotrophic factor.

When adult rodents are subjected to a middle cerebral artery occlusion (MCAO), the resulting infarction injury stimulates a low level of endogenous neurogenesis in the subventricular zone (SVZ) of the affected side (Jin et al., 2001, 2003; Zhang et al., 2001b; Arvidsson et al., 2002; Parent et al., 2002). While this response does not provide for recovery to a pre-stroke condition, it is a promising target for therapeutic intervention since it may be possible to enhance it by adding appropriate factors that increase proliferation, migration and differentiation (PMD), as well as functional integration. Some of the candidate factors (e.g. the epidermal growth factor family EGF; HB-EGF, and erythropoietin, FGF-2, BDNF, G-CSF, G-CSF+ SCF, GDNF, IGF-1, HB-EGF, VEGF) have been shown to produce some enhancement of the endogenous response to acute and sub-acute ischemic stroke (Teramoto et al., 2003; Wang et al., 2004; Wada et al., 2003; Gustafsson et al., 2003; Andsberg et al., 2002; Schneider et al., 2005; Kawada et al., 2006; Dempsey et al., 2003; Jin et al., 2004; Sun et al., 2003). In the present study we investigated whether intracerebral infusion of transforming growth factor-alpha (TGFα), a potent member of the EGF family known to be expressed in the developing and adult forebrain (Fallon et al., 1984; Fallon et al., 1990) can enhance the PMD response in rats subjected to transient MCAO and whether this treatment is associated with significant behavioral recovery. We infused the TGFα 4 weeks after injury when the infarction was no longer expanding and deterioration had stabilized indicating that the acute phase was completed and the chronic phase had begun. This delay in growth factor administration resembles the typical situation in humans with pre-existing stroke injuries who might benefit from this type of therapy.

TGFα is an important cytokine that induces, among other things, the proliferation and differentiation of neural precursors in the developing brain, and low levels of local proliferation in the adult CNS of mammals (Fallon et al., 1984, 1990; Reynolds et al., 1992). In mice that are null for the gene encoding TGFα there are reduced numbers of proliferating SVZ cells especially in the dorsomedial corner of the forebrain lateral ventricle, and fewer neuroblasts in the rostral migratory stream (Tropepe et al., 1997). In a rat model of Parkinson's disease (PD), TGFα induces a massive PMD response by neural stem cells (Fallon et al., 2000) in the ependymal layer and their transit amplifying progeny in the SVZ (Gleason et al., 2008). Some of the daughter cells differentiate as neuron precursors (Gleason et al., 2008) and as neurons associated with significant functional recovery (Fallon et al., 2000).

We find that when TGFα is administered 4 weeks after MCAO, the combined stimuli induce proliferation and mobilization of endogenous precursors that can be clearly detected at 8 weeks after the MCAO. Many of the new cells

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Abbreviations: BrdU, 5′-bromo-2-deoxyuridine; DARRP-32, dopamine and cyclic AMP-regulated phosphoprotein; GFAP, glial fibrillary acidic protein; MCAO, middle cerebral artery occlusion; Meis2, 3′,5′-monophosphate-regulated phosphoprotein; NeuN, neuron-specific nuclear protein; PD, Parkinson's disease; PMD, proliferation, migration and differentiation; SVZ, subventricular zone; TGFα, transforming growth factor-alpha.

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express 3′,5′-monophosphate–regulated phosphoprotein, (Meis2) (Toresson et al., 1999), 32 kD dopamine and cyclic AMP-regulated phosphoprotein (DARPP-32) (Ouimet et al., 1998), and NeuN (Mullen et al., 1992) indicating that they differentiate into striatal neurons appropriate to this brain region. The newly generated neurons appear to become functionally integrated, perhaps by the reconstitution of cortical and basal ganglia circuitry, as measured by standard cylinder and corner behavioral tests (Li et al., 2004; Zhang et al., 2002; Lindner et al., 2003; Schallert et al., 2000). These findings demonstrate the potential efficacy of TGFα as an inducer of neurogenesis, which may be clinically useful in enhancing recovery from neural injuries including stroke.

EXPERIMENTAL PROCEDURES

Transient MCAO

The animals were anesthetized with xylazine (8 mg/kg) and ketamine (100 mg/kg) (Western Medical Supply, Arcadia, CA, USA) and body temperature was maintained with a heating pad set to 37.5 °C. A femoral artery catheter was used for continuous monitoring of mean arterial blood pressure and the measurement of arterial blood gases before, during, and after ischemia. To induce ischemia an incision was made in the neck and the left common, external and internal, carotid arteries were exposed. The external carotid, the left occipital artery and the left pterygopalatine artery were cauterized. A 4–0 nylon monofilament suture with silicon (Doccol Co., Albuquerque, NM, USA) was carefully introduced via the left external carotid artery stump into the lumen of the internal carotid artery until it reached and occluded the MCAO. A surgical clip (Fine Science Tools, Foster City, CA, USA) was placed on the lower part of the internal carotid artery during the time of the occlusion. After 90 min the suture was withdrawn from the internal carotid artery and the wound closed. For sham control animals, the same surgery procedure was used except that no suture was inserted.

Growth factor infusions

Twenty-eight days after the MCAO or sham MCAO procedure the animals were anesthetized with i.p. xylazine (8 mg/kg) and ketamine (100 mg/kg) (Western Medical Supply) and immobilized in a Kopf stereotaxic device. Model (2004), Alzet, Inc. (Brain Infusion Kit), osmotic minipumps were filled with approximately 200 μl of either PBS for control animals, or 20 μg TGFα (R&D Systems, Minneapolis, MN, USA). The pumps were incubated overnight at 37 °C. Minipumps were implanted s.c. in the interscapular region and the 5 mm cannula stereotaxically implanted into the caudate-putamen (A/P −0.8, M/L +3.7, D/V 5), using the bregma as a reference. The cannula was fixed in place using carboxylate cement (International Dental Supply). The infusate was delivered over a 4-week period at a rate of approximately 0.25–1.0 μl/h. A total of 38 animals were given the MCAO, 20 of those were given TGFα, while 18 were given PBS. Eighteen control shams received intraventricular TGFα (n=10), or PBS (n=8). Parts of the damaged striatal tissue sometimes disintegrated and were not available for analysis. All animal experiments were conducted in accordance with applicable oversight guidelines for the ethical use of animals and care was taken to minimize animal suffering.

5′-Bromo-2-deoxyuridine (BrdU) administration

BrdU at 50 mg/kg (Boehringer Mannheim, Mannheim, Germany) was administered intraperitoneally, daily for 28 days.

Table 1. Time course of the experiment

Days	Procedure
1–3	Baseline behavioral experiments for cylinder and corner test
13	MCAO Stroke induction
14, 21, 28, 35	Cylinder and corner tests
43	Minipump implant with TGFα or PBS (28-day infusion) and BrdU injection
50, 57, 64, 71	Cylinder and corner tests, daily BrdU injections for 28 days
72	Sacrifice for histology

MCAO was given to 90-day-old rats to simulate the effects of a stroke injury in adult human patients. Twenty-eight days later TGFα administration was initiated and continued for another 28 days. I.p. daily BrdU administration was initiated on the same day as the TGFα infusion to follow the proliferation of precursor cells. Behavioral tests were given prior to the MCAO procedure, after the MCAO procedure but before the TGFα was administered, and after administration of TGFα. The animals were sacrificed for immunohistochemical studies 56 days after the MCAO procedure and 28 days after the onset of TGFα administration.

Behavioral testing

Animals were assessed for changes in behavioral performance using two sensory-motor tests: the corner test and the cylinder test. The tests were performed once per week on the days stated in Table 1. An average of the three trials was taken. Trends were noted over the course of the experiment and significance of difference between experimental groups was assessed by the Student's t-test and ANOVA test. Post-surgery rats that did not exhibit substantial behavioral deficits in the corner or cylinder tests (~60% of the rats) were, by experimental design, eliminated from further behavioral and immunohistochemical analysis. Also, 2% of the animals were excluded because they became inactive while in the cylinder. This behavior was later attributed to stroke injuries that appeared to be more severe in histological terms than the average injury.

Cylinder test procedure

Trials of the cylinder test were conducted once per week in a room controlled for light and sound to evaluate forelimb use and asymmetry in weight shifting during vertical exploration. Each rat was placed in a transparent cylinder 20 cm in diameter and 30 cm in height with an open top (Interstate Plastics, Sacramento, CA, USA). Counts were made of whether rats supported themselves using the right, left or both forepaws in 10 paw-touches, for a total of 30 noted paw-touches per weekly test. The non-impaired animals used forelimbs symmetrically while MCAO favored one limb over the other. For data analysis, the percent of left (ipsilateral to MCAO lesion) paw use was taken for each trial using the following equation: 100 (# ipsilateral paw touch+0.5 (# both paw touch))/(ipsilateral+contralateral+both paw touch).

Corner test methodology

The edges of two Plexiglas boards with dimensions 30×20×0.5 cm^3 (Interstate Plastics) were placed together to form a 30° angle in front of the rat. The two boards were then vibrated and slowly moved towards the rat to encourage the animal to rear and turn to exit at the open end. Each test consisted of 10 trials in which the total number of left and right turns was recorded. Unlesioned animals turned randomly toward either direction while ischemic rats turned toward the non-impaired side. Our results are reported as the percentage of ipsilateral (injury site, left side) turns versus

contralateral turns. The test was conducted a total of three times/day to achieve statistical significance.

Cardiac perfusion

All animals were sacrificed by intracardiac perfusion at 5 months of age, 2 months after the stroke using methods consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Rats were deeply anesthetized using i.p. ketamine (100 mg/kg) and xylazine (8 mg/kg). When the animal was fully anesthetized, a thoracic incision was made and the pericardium opened. A 0.9% saline solution was perfused intracardially, through the left ventricle, and released via an incision in the right atrium. This was followed by perfusion with a 4% paraformaldehyde solution. The brains were extracted and post-fixed at 4 °C in 4% paraformaldehyde, and cryoprotected in 30% sucrose. The brains were then frozen in methyl butane (Sigma, St. Louis, MO, USA) at −20 °C.

Histological studies

The brains were cut in the coronal plane at 40 μm in a cryostat at −20 °C. For the co-localization of BrdU with different developmental markers for PMD, immunohistochemical double staining was performed on free-floating sections. The tissue slices were treated with 50% formamide, and 50% 2× SSC at 65 °C for 1 h to denature the DNA. This was followed by 3 washes in 2× SSC at room temperature, denaturation with 2 M HCl in H_2O at 37 °C for 30 min, neutralization with 0.1 M borate buffer (pH 8.5) for 10 min, followed by a rinse in PBS pH 7.4 during 5 min. Sections were then blocked in 3% horse serum with 0.3% Triton X-100 in PBS for 1 h at room temperature, and probed with a Roche (Nutley, NJ, USA) anti-BrdU mouse monoclonal antibody (1:400, in 0.1% BSA in PBS) or rat monoclonal anti-BrdU (1:100; Accurate Chemical and Scientific Corporation, Westbury, NY, USA). The BrdU antibody was preincubated with the primary antibodies, which included mouse anti-nestin monoclonal antibody (1:100; Chemicon International, Temecula, CA, USA), rabbit anti–glial fibrillary acidic protein (GFAP; 1:2000; Dako, Carpinteria, CA, USA), rabbit anti-Meis2 (1:500, kind gift of Dr. A. Buchberg, Thomas Jefferson University), mouse anti–neuron-specific nuclear protein (NeuN) (1:100; Chemicon International), mouse anti-DARPP-32 (1:20,000, generously donated by Dr. Paul Greengard, The Rockefeller University). The tissues were then incubated overnight at room temperature and subsequently rinsed in PBS. Alexa Fluor secondary fluorescent antibodies (Invitrogen Corporation, Carlsbad, CA, USA) were incubated for 1 h at room temperature, and the prepared sections were washed in PBS and mounted. Epifluorescence microscopy was performed with a Zeiss Axioplan 2 microscope and a Zeiss LSM510-METAL microscope for confocal microscopy. The three-dimensional reconstructions were made in Bitplane-Imaris software.

Stereological analysis of neurogenesis

The number of BrdU, BrdU/DARPP-32, and BrdU/NeuN cells across the dorsolateral region of the lateral ventricle through the ischemic striatum was estimated by stereological counting procedure using the optical fractionator method. A total of six animals, three for MCAO/TGFα and three for MCAO/PBS were unbiasedly analyzed. Six coronal sections of 40 μm at 280 μm interval, starting at +1.2 to −0.44 mm from Bregma (Paxinos and Watson, 1997), were sampled from each brain. These coordinates correspond to the anteromedial lateral ventricle/striatum area of the brain. The counted area was traced using Stereo Investigator–equipped workstation (MicroBrightfield, Inc., Colchester, VT, USA) at 4× magnification in an Olympus DSU Spinning Disk Confocal Microscope (Olympus, Center Valley, PA, USA). All counts were performed at 100× using a 1.4 numerical aperture lens with a high speed CCD camera (Hamamatsu, Bridgewater, NJ, USA). For BrdU/DARPP-32 and BrdU/NeuN labeling, the counting frames were distributed using a scan grid of 300×300 μm and a counting box of 40 by 40 μm. The BrdU single cells were counted in the ependyma and SVZ using a scan grid of 110×110 μm. Since the number of BrdU cells were distributed homogeneously in the shams we only counted one section per brain (+0.70 mm). For all cell counts, section thicknesses were measured throughout the counting grid with use of the microcator attached to the microscope stage and Gundersen coefficients of error (m=1) were always ≤0.10.

Statistics

The data are expressed as mean values±standard deviation (SD). Statistical analysis between groups was accomplished by unpaired Student's *t*-test and a post hoc Bonferroni test. The data passed the Shapiro–Wilk test and Anderson–Darling normality tests included in an ANOVA analyses. The *P*-value for the comparisons is included in the results and/or figures.

RESULTS

Conditions for chronic stroke

Brains subjected to 90 min of MCAO showed substantial regions that were infarcted 24 h after treatment as indicated by their lack of staining for 2,3,5-triphenyltetrazolium chloride, a red stain that detects active mitochondrial oxidative phosphorylation enzymes in living tissue (Bederson et al., 1986) (Fig. 1A). Areas in the lateral striatum and adjacent cortices where the stroke injury was most severe often showed large regions devoid of tissue (Fig. 1B, C). Although there is no widely accepted time course for assessing the development of MCAO injuries, in human patients the chronic phase begins 1–2 weeks after the stroke and in the present study the lesion sites appeared to have stabilized after 2 weeks. We therefore began administration of TGFα at 4 weeks after occlusion (approximately equivalent to a year after stroke in humans) to ensure that our study was carried out during the chronic phase.

Induction of massive proliferation

In control animals that were given a continuous infusion of PBS and daily administration of BrdU 4 weeks after the MCAO procedure, substantial BrdU incorporation was found in and around the site of the infarction and to a lesser degree in the SVZ of the ipsilateral side at 8 weeks after the infarct (Fig. 1B, D, F). A faint band of BrdU-labeled cells stretched from the dorsolateral corner of the ventricle along the CC and external capsule, including the adjacent subcapsular boundary zone with the striatum (obliterated, embryonic remnant of the lateral ventricle), towards the site of the infarct. However the injury location and ventricle did not appear to be strongly connected by BrdU-labeled cells (Fig. 1B, D, F).

In experimental animals subjected to MCAO and infused with TGFα, there was similarly strong BrdU incorporation in and around the site of the infarction but substantially greater BrdU incorporation along the CC and lateral wall of the ipsilateral ventricle compared to controls (Fig. 1C, E, G). Strong labeling was observed along the lateral wall of the ipsilateral ventricle in both the ependymal layer

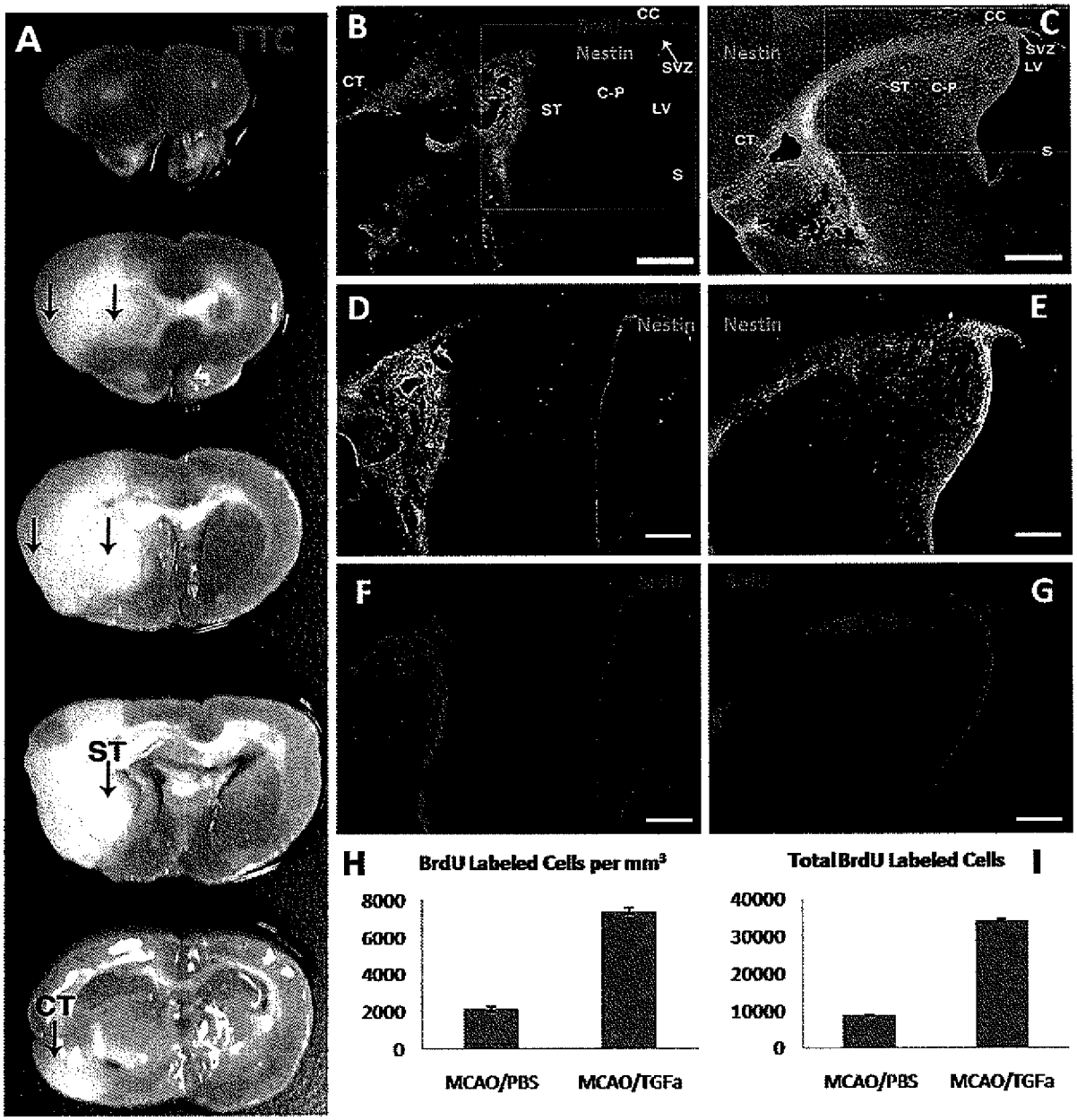

Fig. 1. Histology of MCAO brains and induction of proliferation. (A) Representative coronal brain sections from rats subjected to MCAO for 90 min and stained with 2,3,5-triphenyltetrazolium chloride (TTC) 24 h later. TTC staining shows that the two areas principally affected by the MCAO are the cortex (CT, especially involving the piriform, perirhinal, and suprarhinal cortices) and striatum (ST; and immediately adjacent parts of the globus pallidus). (B, D, F) images of coronal brain sections in adult rats that received a 28-day infusion of PBS, or (C, E, G) TGFα, 28 days after MCAO. (D, F) Magnification of the rectangle in B. (E, G) magnification of the rectangle in C. (H, I) quantification of BrdU-labeled cells in terms of both cells per millimeter cubed and the total number of cells. Scale bars=500 μm (B); 200 μm (C–G). LV, lateral ventricle; C-P caudate putamen; CC, corpus callosum; S, septum; ST, striatum.

and SVZ (Fig. 1G). A manual count of the number of BrdU-labeled cells showed ~487,000 on the ipsilateral side in TGFα-treated animals compared to 228,273 on the same side in PBS controls. Using automated counting of 41 sections we found 34,045 labeled cells on the ipsilateral side in TGFα-treated animals and 8645 on the same side in PBS-treated controls (Fig. 1H, I). We attribute the difference in counts between manual and automated counting here and subsequently to the increased powers of discrimination of the human counter compared to the automated system.

Many of the BrdU-labeled cells were also positive for the neural progenitor marker nestin (Lendahl et al., 1990) which was strongly expressed at the site of the infarct in

both control and experimental animals (Fig. 1B, C, D, E). Nestin expression along the CC was similar to BrdU incorporation, with control animals showing only a partial bridge between the infarct and ventricle whereas TGFα-treated animals showed a more complete bridge (Fig. 1C, E, G). Nestin expression in the SVZ was also substantially greater in treated animals compared to the controls, with expression around the ventricle extending more laterally than BrdU-labeling into the striatum, resulting in a web-like appearance that connected the ventricle wall to more distal parts of the CC where it coursed in a ventral direction (Fig. 1C, E). Although there were some BrdU-labeled cells in the striatal area between the CC and ventricle, most of the co-labeled cells were along the ventricular wall and in the CC (Fig. 1C, E, G). BrdU and nestin on the contralateral side were significantly weaker than on the ipsilateral side and this difference was clearest in experimental animals where the treated side was strongly activated (Fig. 2).

Migration of progenitors

In most TGFα-treated animals the MCAO infarct was located in the lateral parts of the striatum and the deep layers of the lateral cortices. In these animals the BrdU/nestin co-labeled cells were situated along the CC in a band extending from the dorsolateral corner of the ventricle to the site of the infarction. The cells showed nestin staining with bipolar, elongated morphology typical of migrating cells (Fig. 2A, C, E, G, H). In the CC, the long axes of bipolar profiles were oriented approximately orthogonally to the ventricle wall and appeared to point toward the site of the infarction (Fig. 2A, C, G) while along the lateral wall of the ipsilateral ventricle, some cells and their nestin-positive fibers were oriented at ~45° relative to the ventricle surface and appeared to point in the direction of the dorsolateral corner of the ventricle (Fig. 2H). Nestin expression was weaker on the contralateral side where bipolar morphology and potential migratory routes were not observed (Fig. 2B, D, F).

In some animals the infarction was located more medially within the striatum and a different apparent migration pattern was observed (Fig. 3A–L). Here, BrdU-labeled cells were found dispersed throughout the striatum along the entire dorsoventral length of the lateral ventricle, and there were significantly fewer cells in the CC compared to brains with more laterally located infarcts (Fig. 3A, B, C). Expression of doublecortin was dispersed in the striatum between the dorsal extent of the lateral ventricle and the infarct and appeared to increase in the striatum closer to the infarct (Fig. 3A, I). GFAP was also concentrated at the infarct and distributed along the CC leading back to the SVZ (Fig. 3B, J).

Differentiation of progenitors

Meis2 is a transcription factor that is strongly expressed in striatal precursors during development (Toresson et al., 1999) and is thus a marker of the early striatal precursor phenotype. In TGFα-treated animals BrdU-positive cells expressing Meis2 were substantially more common at the infarct, in the apparent migratory stream leading to the infarct (Fig. 3C, G, K) and around the ipsilateral ventricle (Fig. 4B, D) compared to controls (Fig. 3D, H L and Fig. 4A, C). The expression of Meis2 generally resembled that of nestin in forming a bridge that connected the infarct to the lateral ventricle (Fig. 3C, G, K). Nestin, GFAP, doublecortin were also substantially co-localized with Meis2 in the migratory stream and at the infarct (Fig. 3). Meis2 staining in the ependymal layer changed dramatically in treated animals from being concentrated at the ventricle surface to being more evenly distributed among both ependymal cells and more basally located cells in the SVZ.

Labeling with NeuN, a marker of mature neurons showed that while many cells were positive near the lateral ventricles in TGFα-treated animals, cells double-labeled with both BrdU and NeuN were predominantly found at the site of the infarct (Fig. 4F). Closer examination by serial sections of one NeuN positive cell showed both incorporation of BrdU label and distinctive neuronal phenotype in the form of a basal process (Fig. 5A–P). We found many examples of NeuN and BrdU double-labeled cells (Fig. 6C, F, I, L). Manual counting revealed 97,462 cells co-labeled by BrdU and NeuN, while automated counting found a total of 5396 cells (Fig. 6M). In control animals we manually counted 54,854 while automated counting found 696 co-labeled cells in control animals (Fig. 6M). The difference between the counts in control and experimental animals was highly significant.

Many BrdU-labeled cells were also positive for Meis2 and DARPP-32 (Fig. 6) a protein known to be expressed in mature striatal neurons (Ouimet et al., 1998). We found 160,444 BrdU, DARRP-32 co-labeled cells in a manual count of the entire striatum on the ipsilateral side in TGFα-treated animals while automated counting of the same region found 6926 co-labeled cells (Fig. 6M). Manual counting of the entire striatum on the ipsilateral side in control animals identified 69,645 cells while automated counting found 506 co-labeled cells (Fig. 6M), a highly significant difference.

Behavioral recovery

The corner and cylinder sensory-motor behavioral tests (Li et al., 2004; Zhang et al., 2002; Lindner et al., 2003; Schallert et al., 2000) were used to measure asymmetric behavioral preferences that indicate neurological deficiencies related to stroke damage. Using the corner test we found a 98% decrease ($P<0.001$) compared to pre-treatment levels in the frequency of left turns in the stroke animals that were treated with TGFα (Fig. 7A) while animals that received PBS showed a 37% decrease ($P<0.001$) compared to pre-treatment levels. The TGFα-treated rats showed a 61.5% decrease in rotation frequency compared to the PBS stroke-animals 71 days after the stroke and this difference was highly significant ($P<0.01$). The sham-injured rats receiving TGFα or PBS did not show any behavioral improvement ($P<0.001$). Results using the cylinder test were similar to that of the corner test with the TGFα group showing 99% ($P<0.001$) improvement compared to pre-treatment levels, while the

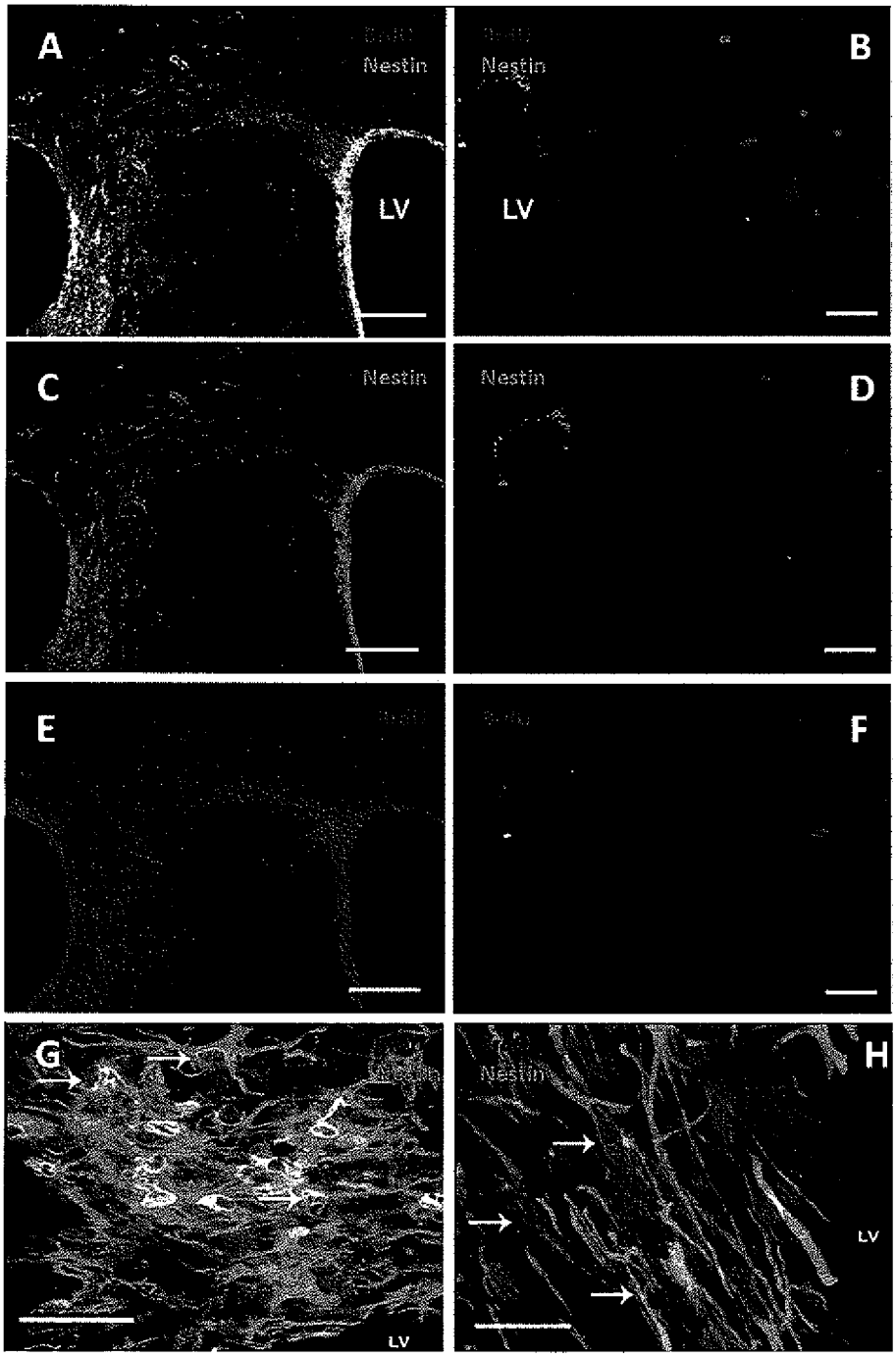

Fig. 2. Migration of activated cells. (A, C, E) The migratory route from the SVZ to the site of the infarct via the corpus callosum in a brain treated with TGFα for 28 days. (B, D, F) The contralateral side where a similar migratory route is not observed. (G, H) Migratory morphology of BrdU/nestin-labeled cells in the dorsolateral corner of the ipsilateral ventricle. (G) Corresponds to the corpus callosum where the cells have a horizontal orientation relative to the ventricle and (H) where cells are oriented at approximately 45° relative to the ventricle surface. Scale bars=250 μm (A, C, E); 200 μm (B, D, F); 20 μm (G, H). LV, lateral ventricle.

PBS-treated stroke group exhibited only 36% ($P<0.001$) improvement (Fig. 7B). The TGFα-treated rats showed a 64% decrease in asymmetric paw placement compared to the PBS stroke-animals 71 days after the stroke and this difference was again highly significant ($P<0.01$). As in the corner test we did not observe any behavioral improvement in sham-treated animals ($P<0.001$). The "hidden platform" version of the Morris water maze test

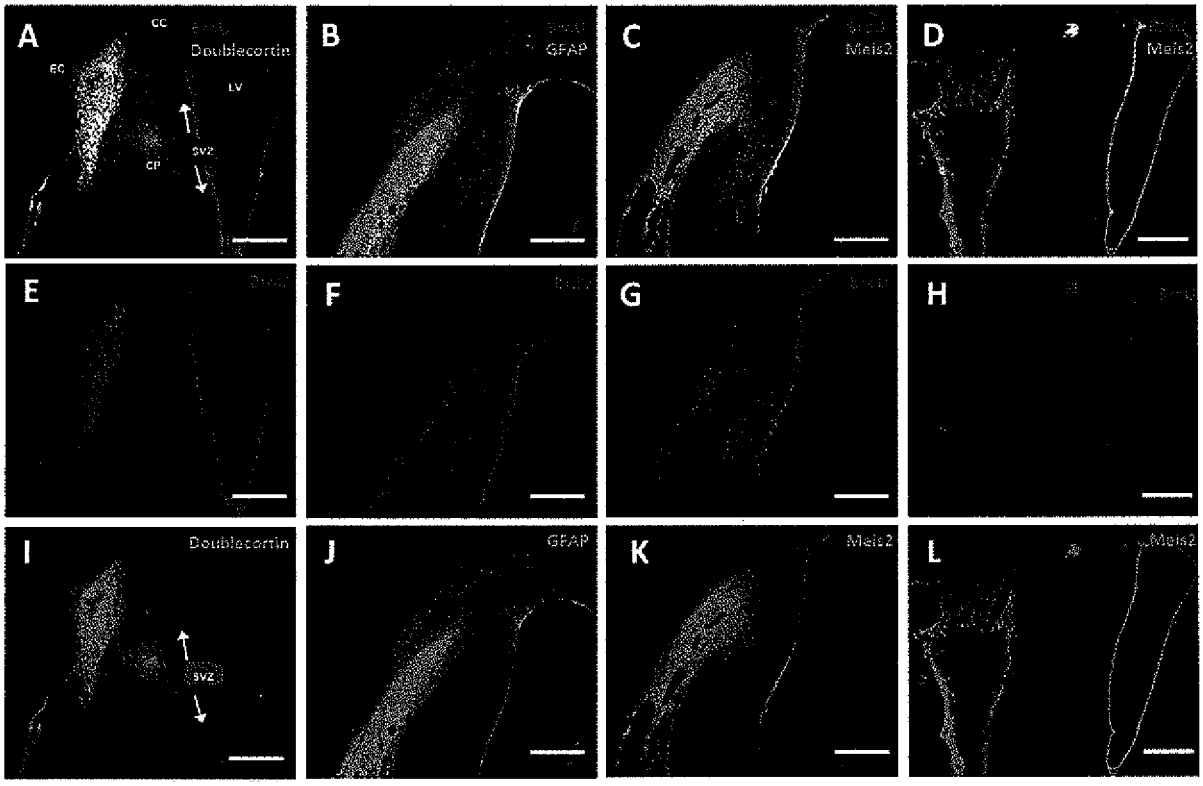

Fig. 3. Differentiation of activated cells. (A, E, I) Doublecortin and BrdU ipsilateral to the infarct in TGFα-treated animals. Expression increases gradually between the ventricle and the infarct but is strongest at the site of the infarction (B, F, J) GFAP is expressed at considerable levels in the same areas in TGFα-treated animals. (C, G, K) Meis2 and BrdU signal are significantly increased in animals treated by TGFα both at the infarct and in a dorsoventrally oriented band of cells leading to it compared to PBS controls (D, H, L). Scale bars=200 μm (A–L). LV, lateral ventricle; C-P, caudate putamen; CC, corpus callosum; SVZ, subventricular zone; EC, external capsule.

(Morris et al., 1982) was used as a negative control to confirm that the measured behavioral deficits were not caused by spatial learning and memory deficits, since these are hippocampal functions not normally affected by the MCAO. No significant changes were found in any of these tests.

DISCUSSION

Here we extend upon our previous work with TGFα and chemical lesion in a PD model rat (Fallon et al., 2000; Gleason et al., 2008) to show that with a chronic stroke/ischemic lesion, TGFα can also significantly amplify the proliferative responses of neural stem cells in the ependymal layer as well as their presumptive progeny in the SVZ (Gleason et al., 2008). Our findings suggest that injury signals in general, rather than signals unique to a particular injury, prime a population of endogenous cells to proliferate in response to appropriate growth factor stimulation, in this case, an epidermal growth factor family member, TGFα, which is the family member enriched in the basal ganglia (Fallon et al., 1990). Newly generated cells then migrate along injury-specific pathways and, in this stroke model, differentiate into neurons in large numbers in association with significant behavioral recovery.

In the absence of TGFα the majority of responding cells, positive for both BrdU and nestin, are found in and around the site of the infarction injury. However, with administration of TGFα most of the additional responding cells are located in the ventricular zone adjacent to the lateral ventricle. After 4 weeks of BrdU administration, labeling of this area was almost complete indicating that a large fraction of adult-germinal cells retain the ability to proliferate and that most of these cells self-renew within a month. The fact that significant proliferation was induced, even when growth factor was not administered until 4 weeks, and in three rats more than 8 weeks (data not shown), after the MCAO injury, indicates that the activated state persists for an extended period after the acute phase of the injury has passed. Administration of TGFα without MCAO does not produce a similar response at the ipsilateral ventricle, demonstrating that the injury provides a signal that is essential for the resulting proliferation, a finding also seen in the PD model rat (Fallon et al., 2000; Gleason et al., 2008; Cooper and Isacson, 2004).

The identity of the BrdU-labeled cells as neural progenitors is confirmed by their expression of nestin (Lendahl et al., 1990). Nestin expression is strongly upregulated in response to injury (Frisen et al., 1995; Takahashi et al.,

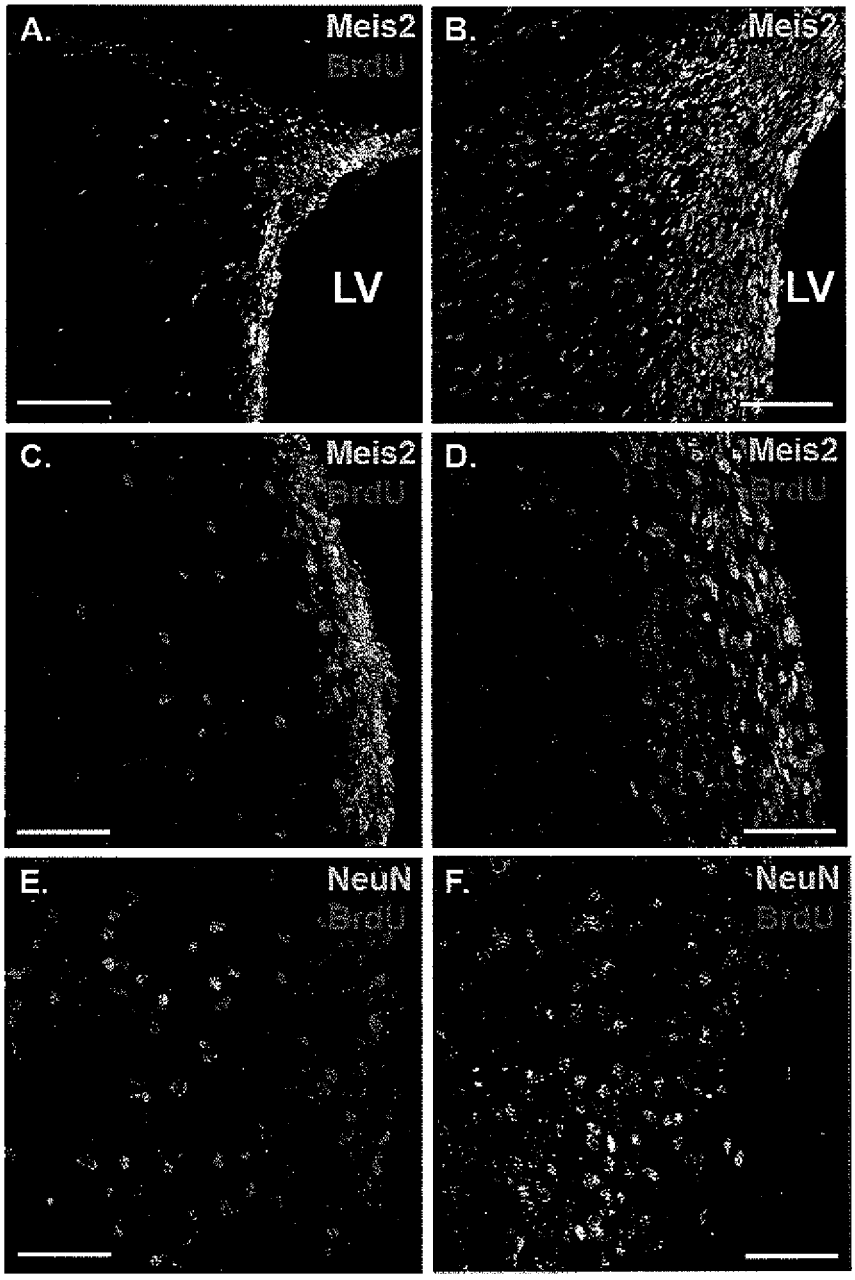

Fig. 4. Distribution of neuronal markers. (A, C) Meis2 and BrdU at the ipsilateral ventricle in PBS-treated animals. Meis2 is accumulated on the ependymal layer and apical SVZ while BrdU incorporation is sporadic. (B, D) Meis2 and BrdU at the ipsilateral ventricle in TGFα-treated animals. BrdU incorporation is dramatically increased compared to controls and the number of cells labeled by either BrdU or Meis2 is substantially greater. Meis2 is no longer accumulated at the ventricle surface and is now distributed more evenly in the SVZ and striatum. (E, F) Localization of NeuN and BrdU at the ventricle surface (E) and at the MCAO site in the striatum (F) in TGFα-treated animals. Very little co-localization is observed near the ventricle even though both markers are prevalent. However at the site of the lesion a substantial fraction of the NeuN positive cells also incorporate BrdU. Scale bars=100 μm (A, B); 50 μm (C–F). LV, lateral ventricle.

2003) and we find significant expansion of the nestin-positive population in MCAO-injured animals treated with TGFα. That nestin was found co-localized with virtually all BrdU-labeled cells indicates that it may be directly involved with cellular proliferation. However, we also found some regions of nestin expression where BrdU incorporation was more limited, suggesting that either a threshold level of expression is required before cells enter a proliferative mode or, alternatively, that nestin has additional functions that are not necessarily connected with cell proliferation.

We presume that the band of BrdU/nestin co-labeled cells that extends from the dorsolateral corner of the ven-

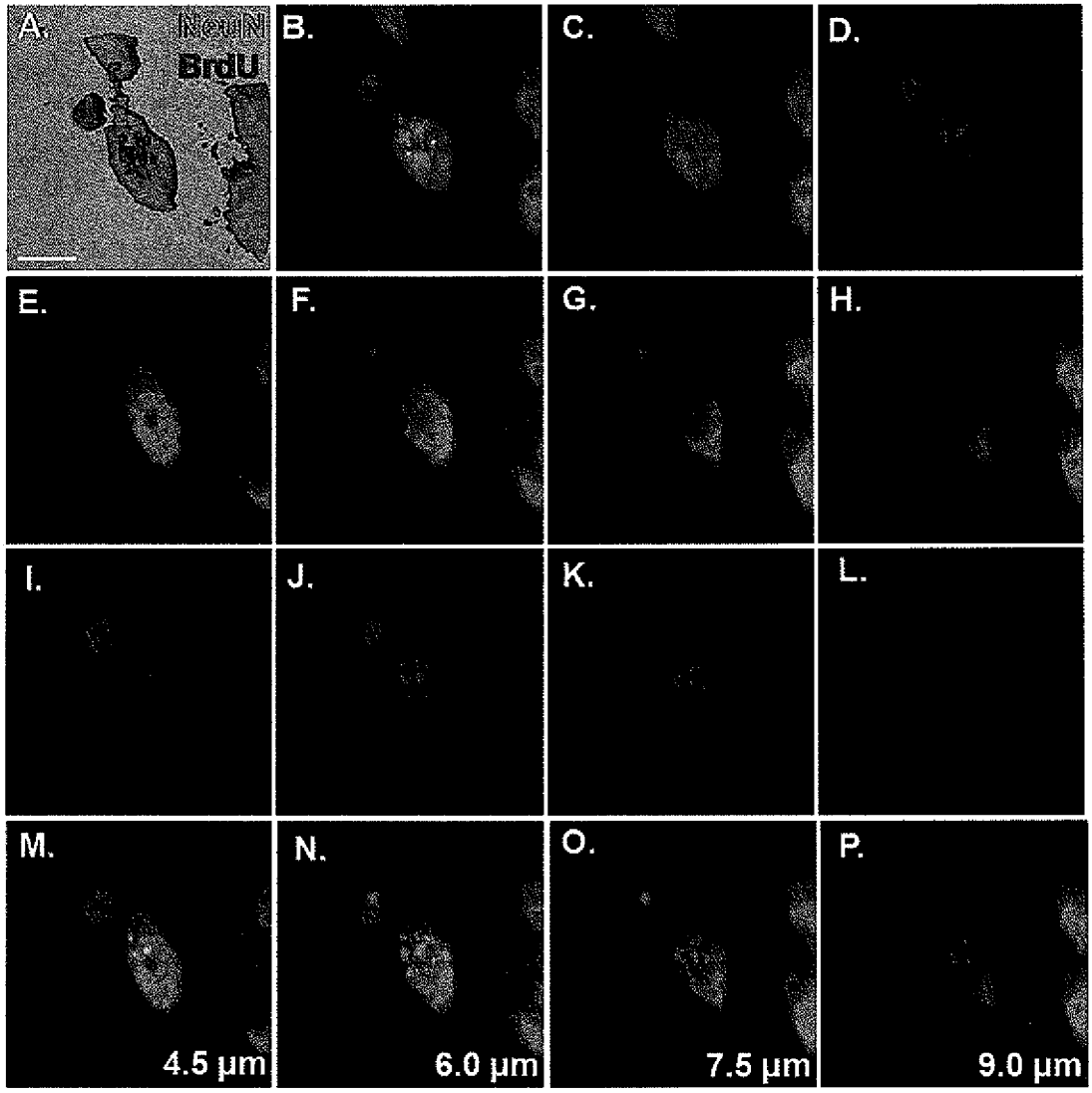

Fig. 5. BrdU-positive cell with neuronal morphology in an MCAO/TGFα-treated animal. (A) A three-dimensional representation of the cell depicted in the remainder of this figure. NeuN staining reveals that the cell has a prominent basal process and substantial BrdU incorporation. (B, C, D) Projections of the entire z-stack into a single plane with both markers (B), NeuN only (C), and BrdU only (D). (E–P) Serial sections through the cell. Scale bar= 10 μm.

tricle along and just ventral to the external capsule to the striatal site of infarction reflects migration of neural precursors from the SVZ to the injury. Despite the fact that traveling through the striatum would be the most direct route from the ventricle to the injury location, we found very few BrdU-labeled cells there when the injury was in the lateral striatum, although nestin was strongly expressed. When the infarct was located more medially within the striatum, BrdU/nestin co-labeled cells appeared to travel directly into the striatum from along the entire dorsoventral length of the SVZ in a more diffuse migratory pathway that was reminiscent of that seen in the PD model (Fallon et al., 2000; Cooper and Isacson, 2004; de Chevigny et al., 2008) where the most acute injury within the forebrain is in the striatum. These findings indicate that injury signals from the lateral extent of the striatum and the cortex are not effectively transmitted through the medial striatum to the SVZ, but must travel through the external capsule, CC, and subcapsular environment while injury signals from more medial locations in the striatum can be directly communicated to the SVZ. In addition when the injury was located in the outer striatum, traveling within the CC migratory pathway was associated with additional proliferation that did not occur in the striatum.

Similar migration patterns have been reported before in stroke models (Arvidsson et al., 2002; Parent et al., 2002; Alonso et al., 1999) and it has been suggested that these migratory flows represent diversions from the default RMS (Parent, 2003). Our finding that doublecortin was expressed increasingly strongly as cells approach the in-

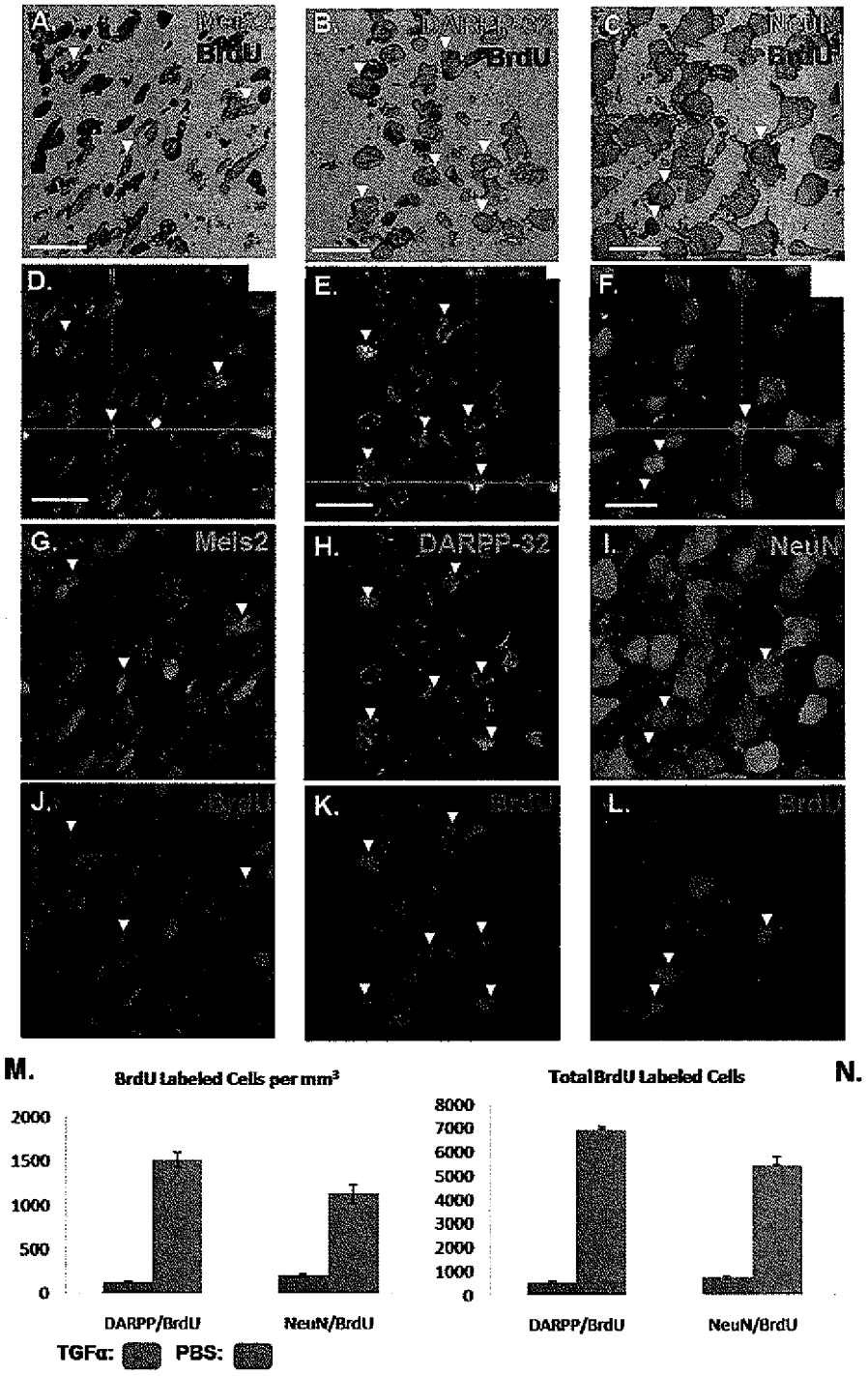

Fig. 6. Neuronal differentiation in MCAO/TGFα-treated animals. Each column in the figure represents one neuronal marker colocalized with BrdU within a single field of cells. (A–C) Three-dimensional representations of each field of cells showing colocalization of the neuron marker with multiple BrdU-positive cells, indicated by arrowheads. The neuron marker (in green) is made partially transparent to facilitate viewing of the inner BrdU-positive nucleus. (D–F) Orthogonal representations from a single plane in each z-stack showing colocalization of one cell at the intersection point of the red and green lines. (G–L) Projections of the entire z-stack into a single plane with each marker. (M, N) Quantification of cells co-labeled by BrdU and a neuron fate marker in terms of both cells per millimeter cubed and the total number of cells. The difference between DARPP/BrdU-, and NeuN/BrdU-labeled cells in both experimental and control animals was significant, $P<0.05$ unpaired t-test, $n=6$. Scale bars=20 μm (A–F). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

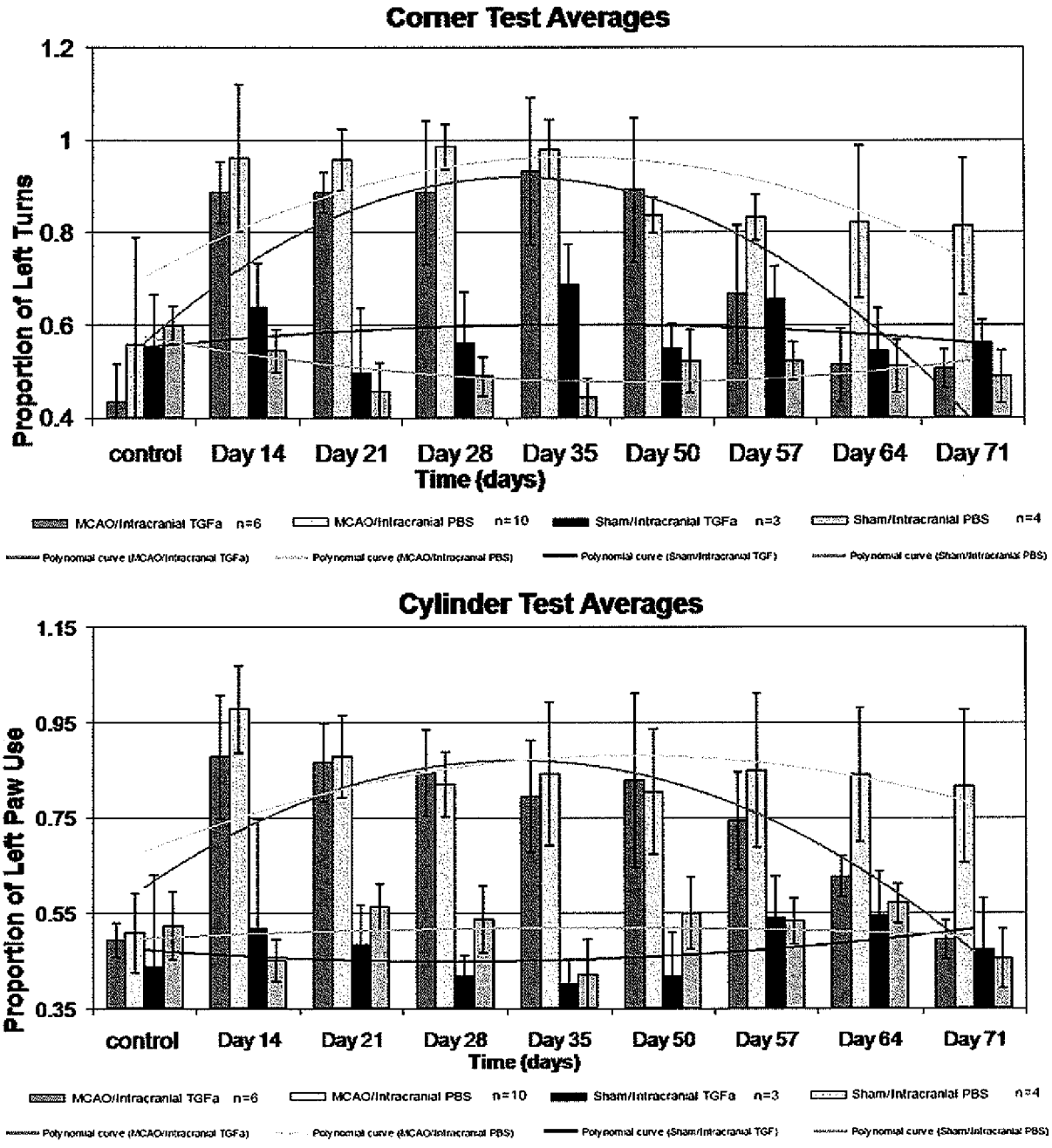

Fig. 7. Behavioral recovery. Mean scores (±SEM) for the groups of rats in both the corner test and cylinder tests before and after the transient MCAO. Values from sham-operated controls (sham/PBS and sham/TGFα) are approximately unchanged over the testing period. In the corner test there is a statistically significant decrease in turning behavior 1 month after growth factor infusion (TGFα) animals at day 14 are significantly different from day 71 (ANOVA test: ** $P<0.001$), also from shams infused with PBS or TGFα (* $P<0.05$). The ANOVA P-value between groups is 1.14E-15. Baseline scores are the averages of three consecutive days of normal, pre-lesion activity at the beginning of the study period). Similarly, in the cylinder test the statistical differences of TGFα animals at day 71 (ANOVA test): ** $P<0$ 0.001 (compared to TGFα at day 14, beginning of the post-injury period) are significant compared to shams infused with PBS or TGFα (* $P<0.05$). The ANOVA P-value between groups is of 2.83E-22.

farct as well as the GFAP expression both in the striatum and CC may be consistent with that concept. However, the intense GFAP expression that we observed at the site of the infarct may be indicative of reactive gliosis and the formation of a glial scar (Buffo et al., 2008; Pekny and Nilsson, 2005). Thus while the proliferative response to TGFα appears to be a generalized phenomenon that is shared in common across two types of neural injury, the

pattern of migration and destination of the newly generated cells is dependent on the injury so that the new cells are traveling to the locations where they are needed along pathways that reflect the location of the injury.

Eight weeks after the MCAO, and 4 weeks after the onset of TGFα administration we found a large number of BrdU-labeled cells expressing neuronal-fate markers, and some of these cells also showed the morphological characteristics of neurons. Many cells also expressed doublecortin suggesting they matured along stereotypical differentiation pathways. High levels of neuronal differentiation have been reported in several previous studies of stroke and other brain injuries, and our results are in general agreement with those reports except that we found greater numbers of neurons (Arvidsson et al., 2002; Parent et al., 2002; Zhang et al., 2001a; Lichtenwalner and Parent, 2006). These results indicate that the TGFα treatment significantly increased the yield of neurons produced in the injury response. Although we did not examine the long-term survival of these neurons, the behavioral data in the chronic animal indicate that many of them became functionally integrated.

It has been reported previously that the systemic administration of TGFα may impair neuronal differentiation, at least in the PD model, perhaps by preventing cells from exiting the cell cycle (Cooper and Isacson, 2004; de Chevigny et al., 2008). This idea seems substantiated by several other studies which report that growth factor causes neural cells to undergo dedifferentiation, including reversion to earlier phenotypic stages, reduced migration and increased proliferation (Doetsch et al., 2002; Sharif et al., 2007). Although these effects probably occur in our MCAO model, they seem to have been outweighed by the propensity of new cells to travel to the injury site and differentiate. Several factors may account for this outcome. First is the delay between MCAO injury and the onset of TGFα administration which distinguishes this model from the PD/TGFα model. This interval between the two stimuli may have provided enough reduction in the overall stimulating effect to allow for greater differentiation than would be possible when the stimuli are contemporaneous. A second possibility is that stroke injuries are more likely to lead to subsequent neurogenesis, compared with injuries resulting from a 6-OHDA lesion. A third factor is that we assessed neuronal differentiation at 8 weeks after the stroke injury, which is later than in many other studies.

Perhaps the most important question in these studies is whether the newly generated neurons can functionally integrate into the adult brain and contribute to behavioral recovery. Our TGFα-treated animals certainly experienced a more complete behavioral recovery (99% on tests designed to evaluate stroke damage) than did animals relying upon only the endogenous response. We presume that since the most significant difference between the control and treated animals appears to be the response elicited by TGFα along the ventricle, these additional cells are probably responsible for the additional behavioral improvement.

It has long been thought that the adult mammalian brain possesses a limited capacity for regeneration, because of the scarcity of proliferating cells and because of the limited ability of adult mammals to show behavioral recovery from brain injuries. However, this view has been revised in recent decades by the discovery of neurogenesis in the SVZ and dentate gyrus (Lichtenwalner and Parent, 2006). Although these regions are thought to supply neuron precursors to well-defined regions (the olfactory bulb and hippocampus respectively) there is increasing evidence that cells on the migratory pathways supplying these areas can be partially diverted to other destinations including the striatum and cortex (Lichtenwalner and Parent, 2006). The evidence also suggests that local precursor cells are present throughout the brain (Reynolds et al., 1992; Sharif et al., 2007) such that a low level of endogenous neurogenesis from persistent germinal zones and dispersed precursors may be possible in the adult (Lichtenwalner and Parent, 2006). Our data clearly show the activity of TGFα in promoting PMD in the CNS after injury in the adult rat brain. Together with the recent discovery that the human brain undergoes postischemic neurogenesis (Jin et al., 2006) the results encourage the idea of TGFα could be used as a therapeutic agent in humans.

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REFERENCES

Alonso G, Prieto M, Chauvet N (1999) Tangential migration of young neurons arising from the subventricular zone of adult rats is impaired by surgical lesions passing through their natural migratory pathway. J Comp Neurol 405:508–528.

Andsberg G, Kokaia Z, Klein RL, Muzyczka N, Lindvall O, Mandel RJ (2002) Neuropathological and behavioral consequences of adeno-associated viral vector-mediated continuous intrastriatal neurotrophin delivery in a focal ischemia model in rats. Neurobiol Dis 9: 187–204.

Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O (2002) Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med 8:963–970.

Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartkowski HM (1986) Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. Stroke 17:1304–1308.

Buffo A, Rite I, Tripathi P, Lepier A, Colak D, Horn AP, Mori T, Gotz M (2008) Origin and progeny of reactive gliosis: A source of multipotent cells in the injured brain. Proc Natl Acad Sci U S A 105: 3581–3586.

Cooper O, Isacson O (2004) Intrastriatal transforming growth factor alpha delivery to a model of Parkinson's disease induces prolifer-

ation and migration of endogenous adult neural progenitor cells without differentiation into dopaminergic neurons. J Neurosci 24: 8924–8931.

de Chevigny A, Cooper O, Vinuela A, Reske-Nielsen C, Lagace DC, Eisch AJ, Isacson O (2008) Fate mapping and lineage analyses demonstrate the production of a large number of striatal neuroblasts after transforming growth factor alpha and noggin striatal infusions into the dopamine-depleted striatum. Stem Cells 26: 2349–2360.

Dempsey RJ, Sailor KA, Bowen KK, Tureyen K, Vemuganti R (2003) Stroke-induced progenitor cell proliferation in adult spontaneously hypertensive rat brain: effect of exogenous IGF-1 and GDNF. J Neurochem 87:586–597.

F, Doetsch Petreanu L, Caille I, Garcia-Verdugo JM, Alvarez-Buylla A (2002) EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. Neuron 36:1021–1034.

J, Fallon Reid S, Kinyamu R, Opole I, Opole R, Baratta J, Korc M, Endo TL, Duong A, Nguyen G, Karkehabadhi M, Twardzik D, Patel S, Loughlin S (2000) In vivo induction of massive proliferation, directed migration, and differentiation of neural cells in the adult mammalian brain. Proc Natl Acad Sci U S A 97:14686–14691.

Fallon JH, Annis CM, Gentry LE, Twardzik DR, Loughlin SE (1990) Localization of cells containing transforming growth factor-alpha precursor immunoreactivity in the basal ganglia of the adult rat brain. Growth Factors 2:241–250.

Fallon JH, Seroogy KB, Loughlin SE, Morrison RS, Bradshaw RA, Knaver DJ, Cunningham DD (1984) Epidermal growth factor immunoreactive material in the central nervous system: location and development. Science 224:1107–1109.

Frisen J, Johansson CB, Torok C, Risling M, Lendahl U (1995) Rapid, widespread, and longlasting induction of nestin contributes to the generation of glial scar tissue after CNS injury. J Cell Biol 131: 453–464.

Gleason D, Fallon JH, Guerra M, Liu JC, Bryant PJ (2008) Ependymal stem cells divide asymmetrically and transfer progeny into the subventricular zone when activated by injury. Neuroscience 156: 81–88.

Gustafsson E, Andsberg G, Darsalia V, Mohapel P, Mandel RJ, Kirik D, Lindvall O, Kokaia Z (2003) Anterograde delivery of brain-derived neurotrophic factor to striatum via nigral transduction of recombinant adeno-associated virus increases neuronal death but promotes neurogenic response following stroke. Eur J Neurosci 17:2667–2678.

Jin K, Minami M, Lan JQ, Mao XO, Batteur S, Simon RP, Greenberg DA (2001) Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. Proc Natl Acad Sci U S A 98:4710–4715.

Jin K, Sun Y, Xie L, Childs J, Mao XO, Greenberg DA (2004) Post-ischemic administration of heparin-binding epidermal growth factor-like growth factor (HB-EGF) reduces infarct size and modifies neurogenesis after focal cerebral ischemia in the rat. J Cereb Blood Flow Metab 24:399–408.

Jin K, Sun Y, Xie L, Peel A, Mao XO, Batteur S, Greenberg DA (2003) Directed migration of neuronal precursors into the ischemic cerebral cortex and striatum. Mol Cell Neurosci 24:171–189.

Jin K, Wang X, Xie L, Mao XO, Zhu W, Wang Y, Shen J, Mao Y, Banwait S, Greenberg DA (2006) Evidence for stroke-induced neurogenesis in the human brain. Proc Natl Acad Sci U S A 103: 13198–13202.

Kawada H, Takizawa S, Takanashi T, Morita Y, Fujita J, Fukuda K, Takagi S, Okano H, Ando K, Hotta T (2006) Administration of hematopoietic cytokines in the subacute phase after cerebral infarction is effective for functional recovery facilitating proliferation of intrinsic neural stem/progenitor cells and transition of bone marrow-derived neuronal cells. Circulation 113:701–710.

Lendahl U, Zimmerman LB, McKay RD (1990) CNS stem cells express a new class of intermediate filament protein. Cell 60:585–595.

Li X, Blizzard KK, Zeng Z, DeVries AC, Hum PD, McCullough LD (2004) Chronic behavioral testing after focal ischemia in the mouse: functional recovery and the effects of gender. Exp Neurol 187:94–104.

Lichtenwalner RJ, Parent JM (2006) Adult neurogenesis and the ischemic forebrain. J Cereb Blood Flow Metab 26:1–20.

Lindner MD, Gribkoff VK, Donlan NA, Jones TA (2003) Long-lasting functional disabilities in middle-aged rats with small cerebral infarcts. J Neurosci 23:10913–10922.

Morris RG, Garrud P, O' Rawlins JN, Keefe J (1982) Place navigation impaired in rats with hippocampal lesions. Nature 297:681–683.

Mullen RJ, Buck CR, Smith AM (1992) NeuN, a neuronal specific nuclear protein in vertebrates. Development 116:201–211.

Ouimet CC, Langley-Gullion KC, Greengard P (1998) Quantitative immunocytochemistry of DARPP-32-expressing neurons in the rat caudatoputamen. Brain Res 808:8–12.

Parent JM (2003) Injury-induced neurogenesis in the adult mammalian brain. Neuroscientist 9:261–272.

Parent JM, Vexler ZS, Gong C, Derugin N, Ferriero DM (2002) Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. Ann Neurol 52:802–813.

Pekny M, Nilsson M (2005) Astrocyte activation and reactive gliosis. Glia 50:427–434.

Reynolds BA, Tetzlaff W, Weiss S (1992) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci 12:4565–4574.

Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST (2000) CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, Parkinsonism and spinal cord injury. Neuropharmacology 39:777–787.

Schneider A, Kruger C, Steigleder T, Weber D, Pitzer C, Laage R, Aronowski J, Maurer MH, Gassler N, Mier W, Hasselblatt M, Kollmar R, Schwab S, Sommer C, Bach A, Kuhn HG, Schabitz WR (2005) The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. J Clin Invest 115:2083–2098.

Sharif A, Legendre P, Prevot V, Allet C, Romao L, Studler JM, Chneiweiss H, Junier MP (2007) Transforming growth factor alpha promotes sequential conversion of mature astrocytes into neural progenitors and stem cells. Oncogene 26:2695–2706.

Sun Y, Jin K, Xie L, Childs J, Mao XO, Logvinova A, Greenberg DA (2003) VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. J Clin Invest 111: 1843–1851.

Takahashi M, Arai Y, Kurosawa H, Sueyoshi N, Shirai S (2003) Ependymal cell reactions in spinal cord segments after compression injury in adult rat. J Neuropathol Exp Neurol 62:185–194.

Teramoto T, Qiu J, Plumier JC, Moskowitz MA (2003) EGF amplifies the replacement of parvalbumin-expressing striatal interneurons after ischemia. J Clin Invest 111:1125–1132.

Toresson H, Mata de Urquiza A, Fagerstrom C, Perlmann T, Campbell K (1999) Retinoids are produced by glia in the lateral ganglionic eminence and regulate striatal neuron differentiation. Development 126:1317–1326.

Tropepe V, Craig CG, Morshead CM, van der Kooy D (1997) Transforming growth factor-alpha null and senescent mice show decreased neural progenitor cell proliferation in the forebrain subependyma. J Neurosci 17:7850–7859.

Wada K, Sugimori H, Bhide PG, Moskowitz MA, Finklestein SP (2003) Effect of basic fibroblast growth factor treatment on brain progenitor cells after permanent focal ischemia in rats. Stroke 34:2722–2728.

Wang L, Zhang Z, Wang Y, Zhang R, Chopp M (2004) Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. Stroke 35:1732–1737.

Zhang L, Schallert T, Zhang ZG, Jiang Q, Arniego P, Li Q, Lu M, Chopp M (2002) A test for detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischemia. J Neurosci Methods 117:207–214.

Zhang RL, Zhang ZG, Zhang L, Chopp M (2001a) Proliferation and differentiation of progenitor cells in the cortex and the subventricular zone in the adult rat after focal cerebral ischemia. Neuroscience 105:33–41.

Zhang RL, Zhang ZG, Zhang L, Chopp M (2001b) Proliferation and differentiation of progenitor cells in the cortex and the subventricular zone in the adult rat after focal cerebral ischemia. Neuroscience 105:33–41.

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EXHIBIT G

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Epidermal Growth Factor and Fibroblast Growth Factor-2 Have Different Effects on Neural Progenitors in the Adult Rat Brain

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Neurons and glia are generated throughout adulthood from proliferating cells in two regions of the rat brain, the subventricular zone (SVZ) and the hippocampus. This study shows that exogenous basic fibroblast growth factor (FGF-2) and epidermal growth factor (EGF) have differential and site-specific effects on progenitor cells *in vivo*. Both growth factors expanded the SVZ progenitor population after 2 weeks of intracerebroventricular administration, but only FGF-2 induced an increase in the number of newborn cells, most prominently neurons, in the olfactory bulb, the normal destination for neuronal progenitors migrating from the SVZ. EGF, on the other hand, reduced the total number of newborn neurons reaching the olfactory bulb and substantially enhanced the generation of astrocytes in the olfactory bulb. Moreover, EGF increased the number of newborn cells in the striatum either by migration of SVZ cells or by stimulation of local progenitor cells. No evidence of neuronal differentiation of newborn striatal cells was found by three-dimensional confocal analysis, although many of these newborn cells were associated closely with striatal neurons. The proliferation of hippocampal progenitors was not affected by either growth factor. However, EGF increased the number of newborn glia and reduced the number of newborn neurons, similar to the effects seen in the olfactory bulb. These findings may be useful for elucidating the *in vivo* role of growth factors in neurogenesis in the adult CNS and may aid development of neuronal replacement strategies after brain damage.

Key words: subventricular zone; hippocampus; epidermal growth factor; basic fibroblast growth factor; intracerebroventricular administration; progenitor cells; stem cells; proliferation; neurogenesis; gliogenesis

The adult CNS appears to have only limited potential to generate new neurons, making it vulnerable to injury and disease. However, certain areas of the brain retain the capacity for neurogenesis well into adulthood (Altman and Das, 1965; Kuhn et al., 1996). In the adult rodent a rapidly dividing population of stem cells in the subventricular zone (SVZ) of the lateral ventricle generates all neural cell types: neurons, astrocytes, and oligodendrocytes (Lewis, 1968; Privat and Leblond, 1972; Corotto et al., 1993; Lois and Alvarez-Buylla, 1993; Morshead et al., 1994; Goldman, 1995; Hauke et al., 1995). From the SVZ neuronal progenitors migrate tangentially (sagittally) along the rostral migratory stream (RMS) into the olfactory bulb (OB), where they differentiate into granule and periglomerular neurons (Corotto et al., 1993; Lois and Alvarez-Buylla, 1993, 1994; Luskin, 1993; Goldman, 1995; Lois et al., 1996). In contrast, glial progenitor cells migrate radially into neighboring brain structures such as striatum, corpus callosum, and neocortex (Levison and Goldman, 1993; Levison et al., 1993; Luskin and McDermott, 1994). In the hippocampal dentate gyrus of adult rats, neural precursor cells continue to proliferate and differentiate into granule cells (Kaplan and Hinds, 1977; Kaplan and Bell, 1983; Cameron et al., 1993; Kuhn et al., 1996).

To be able to manipulate the endogenous adult progenitors, we believe it is crucial to determine the extracellular signals that can stimulate cell division and regulate the fate of these neural stem and progenitor cells (Cattaneo and McKay, 1991; Gage, 1994). Recently, several groups successfully have isolated and propagated adult neural progenitor cells *in vitro* (Reynolds and Weiss, 1992; Richards et al., 1992; Lois and Alvarez-Buylla, 1993; Vescovi et al., 1993; Morshead et al., 1994; Gage et al., 1995a; Palmer et al., 1995; Gritti et al., 1996). Dissociated cells from the SVZ and the hippocampus required basic fibroblast growth factor (FGF-2) or epidermal growth factor (EGF) for proliferation and long-term survival *in vitro*. Some of these cells retain the ability to generate both neurons and glia, suggesting that newborn cells of the adult brain may originate from stem cell-like progenitors (Morshead et al., 1994; Gage et al., 1995a; Palmer et al., 1995; Gritti et al., 1996; Suhonen et al., 1996).

The possibility that growth factors also may influence neural progenitors *in vivo* has been supported by findings that intracerebroventricular administration of EGF expanded proliferative progenitors in the SVZ of adult mice (Craig et al., 1996). Numerous newborn cells were found in the adjacent striatum, septum, and cortex, and a small portion of these cells expressed neuronal antigens.

The goal of the present study was to explore systematically the effects of FGF-2 or EGF on the proliferation and differentiation of neural progenitor cells in the SVZ/OB system and the dentate gyrus of adult rats. FGF-2, EGF, or artificial CSF (aCSF) were chronically infused into the lateral ventricle. The proliferative

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H.G.K. and J.W. have contributed equally to this manuscript.

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zones, migratory paths, and areas of neuronal differentiation were analyzed quantitatively for the number and phenotype of newborn cells.

MATERIALS AND METHODS

Animals and surgery

Male Fischer-344 albino rats (n = 30; Harlan Sprague Dawley, Indianapolis, IN) were used in this experiment. The animals were 13–14 weeks old and weighed between 260 and 300 gm at the start of the experiment. Anesthesia was induced by an intramuscular injection consisting of 62.5 mg/kg ketamine (Ketaset, 100 mg/ml, Bristol Laboratories, Syracuse, NY), 3.175 mg/kg xylazine (Rompun, 20 mg/ml, Miles Laboratories, Shawnee, KS), and 0.625 mg/kg acepromazine maleate (10 mg/ml, TechAmerica Group, Elwood, KS) dissolved in 0.9% sterile saline. Rats were mounted in a small animal stereotaxic apparatus (David Kopf, Tujunga, CA) with bregma and lambda in the same horizontal plane. A stainless steel cannula (28 gauge, Plastic Products, Roanoke, VA) was implanted in the lateral ventricle [anteroposterior (AP) +8.5 mm, lateral +1.5 mm from the center of the interaural line in flat skull position; cannula length, 5 mm] and connected by 3.5 cm vinyl tubing (size V/4, Bolab, Lake Havasu City, AZ) to an osmotic minipump (model 2002, Alza, Palo Alto, CA). Human recombinant EGF (30 μg/ml, Promega, Madison, WI) or FGF-2 (30 μg/ml, A. Baird, Prizm Pharmaceuticals, San Diego, CA) was dissolved in aCSF [(in mM): 148 NaCl, 3 KCl, 1.4 $CaCl_2$, 0.8 $MgCl_2$, 1.5 Na_2HPO_4, and 0.2 NaH_2PO_4, pH 7.4] containing 100 μg/ml rat serum albumin (Sigma, St. Louis, MO). An antibiotic (gentamycin, 50 μg/ml, Sigma) was included in the infusate. The animals received EGF (n = 10), FGF-2 (n = 10), or aCSF (n = 10) at a flow rate of 0.50 μl/hr, resulting in a delivery of 360 ng of growth factor per day for 14 d. During the last 12 d of the pump period animals received daily intraperitoneal injections of bromodeoxyuridine (BrdU, 50 mg/kg, Sigma). At the end of the treatment one-half of the animals (n = 5 per group) were anesthetized deeply and perfused intracardially with 4% paraformaldehyde in 100 mM phosphate buffer, pH 7.4. Brains were removed, post-fixed overnight in 4% paraformaldehyde, and transferred to 0.32 M sucrose. In the remaining one-half of the animals the pumps were removed under methoxyflurane anesthesia. The vinyl tubing was ligated with sterile nonabsorbable black monofilament nylon (3–0 Dermalon, American Cyanamid, Danbury, CT). These animals were perfused after an additional 4 week period without growth factor infusion.

Histology

The brains were cut in three parts, providing material for (1) coronal sections of the SVZ and hippocampus and sagittal sections of the (2) OB and (3) cerebellum. Sections (40 μm) were cut with a sliding microtome and stored at −20°C in a cryoprotectant solution (glycerol, ethylene glycol, and 0.1 M phosphate buffer, pH 7.4, 3:3:4 by volume).

Antibodies and immunochemicals. The following antibodies and final dilutions were used: mouse (mo) α-BrdU (1:400, Boehringer Mannheim, Indianapolis, IN), rat α-BrdU (1:100, Accurate, Westbury, NY), mo α-PSA-NCAM (1:2500, clone MenB kindly provided by Dr. G. Rougon, University of Marseille, Marseille, France), mo α-NeuN (1:20, clone A60 kindly provided by Dr. R. Mullen, University of Utah, Salt Lake City, UT), rabbit α-S100β (1:5000, Swant, Bellinzona, Switzerland), biotinylated horse α-mouse IgG (1:160, Vector Laboratories, Burlingame, CA), avidin–biotin–peroxidase complex (1:100, Vectastain Elite, Vector Laboratories), and donkey α-rat–FITC, α-mouse–Texas Red, and α-rabbit–CY5 (all 1:300, Jackson ImmunoResearch, West Grove, PA).

Immunoperoxidase. Free-floating sections were treated with 0.6% H_2O_2 in TBS (0.15 M NaCl and 0.1 M Tris-HCl, pH 7.5) for 30 min to block endogenous peroxidase. For DNA denaturation, sections were incubated for 2 hr in 50% formamide/2× SSC (0.3 M NaCl and 0.03 M sodium citrate) at 65°C, rinsed for 5 min in 2× SSC, incubated for 30 min in 2N HCl at 37°C, and rinsed for 10 min in 0.1 M boric acid, pH 8.5. Several rinses in TBS were followed by incubation in TBS/0.1% Triton X-100/3% normal horse serum (TBS–Ths) for 30 min and incubation with mo α-BrdU antibody in TBS–Ths overnight at +4°C. After being rinsed in TBS–Ths, sections were incubated for 1 hr with biotinylated horse α-mouse antibody. With intermittent rinses in TBS, avidin–biotin–peroxidase complex was applied for 1 hr, followed by peroxidase detection for 5 min (0.25 mg/ml DAB, 0.01% H_2O_2, 0.04% NiCl).

Immunofluorescence. Sections were treated for DNA denaturation as described above, followed by several rinses in TBS and incubation in TBS/0.1% Triton X-100/3% normal donkey serum (TBS–Tds) for 30 min. Primary antibodies were applied in TBS–Tds for 48 hr at +4°C, rinsed in TBS three times for 10 min, and blocked in TBS–Tds for 10 min. Antibodies were detected with donkey α-rat, mouse, or rabbit coupled to FITC, Texas Red, or CY5 for 2 hr. Fluorescent signals were detected and processed by a confocal scanning laser microscope (Bio-Rad MRC1024, Hercules, CA) and Adobe Photoshop (Adobe Systems, Mountainview, CA).

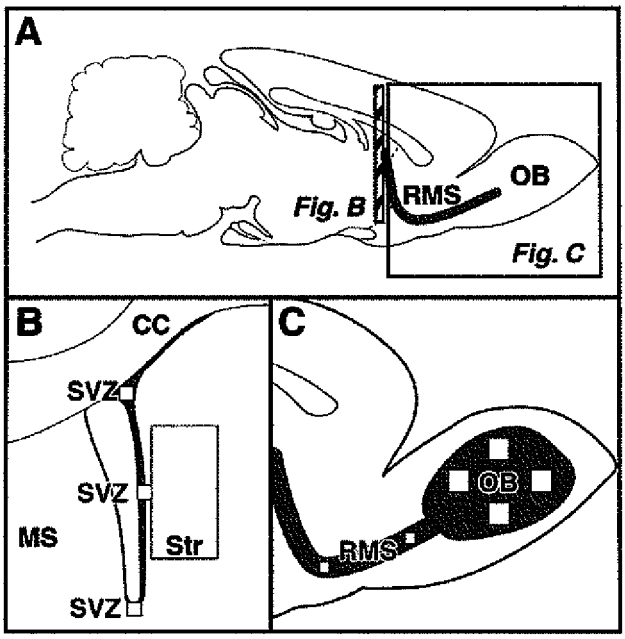


Figure 1. Analysis of the subventricular zone (*SVZ*) and olfactory bulb. *A*, Sagittal view of the rat brain illustrating the anatomical sites of progenitor proliferation in the *SVZ*, migration along the rostral migratory stream (*RMS*), and differentiation in the olfactory bulb (*OB*). *Hatched bar* indicates position of coronal view in *B*. *B*, Coronal plane of the lateral ventricle with the corpus callosum (*CC*), medial septum (*MS*), and striatum (*Str*). Three areas in the *SVZ* (ventral, lateral, and dorsal *squares*, 50 × 50 μm) and one area in the striatum (*large rectangle*, 300 × 600 μm) were analyzed for BrdU-positive cells on each section. *C*, Parasagittal plane of frontal cortex and olfactory bulb. Two areas of the RMS (*small squares*, 50 × 50 μm) and four areas of the *OB* granule cell layer (*large squares*, 100 × 100 μm) were analyzed for BrdU-positive cells and colabeling with NeuN or S100β.

Quantification

Quantification of BrdU-positive cells was accomplished with unbiased counting methods. The optical disector procedure (Sterio, 1984) was used to determine the three-dimensional numerical density of BrdU-positive cells, which is expressed as cells/mm^3. Structures were sampled either by selecting predetermined areas on each section (Fig. 1; SVZ, striatum, RMS, and OB) or by analyzing entire structures on each section (dentate gyrus and cerebellum). In the latter case we used a point-counting grid for determination of the sampling volume via the Cavalieri method (Michel and Cruz-Orive, 1988).

Lateral ventricle and striatum. Every 12th section from a coronal series of the striatum was selected between AP +10.6 mm—genu corpus callosum—and AP +8.74 mm—anterior commissure crossing (Paxinos and Watson, 1986). As illustrated in Figure 1*B*, BrdU-positive cells were counted in three predetermined areas (50 × 50 μm) of the lateral ventricle wall on all selected sections. A rectangular area of the striatum (300 × 600 μm) was selected at a 50 μm distance from the lateral ventricle wall and analyzed on each section. All BrdU-positive nuclei in these selected areas were counted and presented as the number of cells (in thousands)/mm^3 (Table 1).

RMS and OB. Every sixth section (40 μm) from sagittal series of the OB/frontal cortex was selected and stained for BrdU immunohistochem-

Table 1. Density and cell fate of newborn cells in the subventricular zone, olfactory bulb, and striatum

Area	aCSF	FGF-2	EGF
Subventricular zone			
1 d after infusion			
Cannula side	62.2 ± 9.8	208.5 ± 62.2**	595.5 ± 133.1**
Contralateral side	37.1 ± 8.1	32.6 ± 4.4	70.3 ± 9.9**
4 weeks after infusion			
Cannula side	30.6 ± 10.7	104.8 ± 27.7**	153.6 ± 27.8**
Contralateral side	13.3 ± 3.9	13.5 ± 2.7	88.0 ± 11.8**
Rostral migratory str.			
1 d after infusion	915.5 ± 50.0	714.4 ± 38.0*	368.8 ± 25.0**
Olfactory bulb			
4 weeks after infusion			
Total	40.9 ± 2.0 (100)	46.9 ± 1.2* (100)	16.3 ± 1.6** (100)
Neurons	39.3 ± 1.6 (96)	45.2 ± 0.9* (96)	11.8 ± 1.5** (72)
Astrocytes	0.16 ± 0.11 (<0.1)	0.26 ± 0.10 (<0.1)	2.26 ± 0.54** (14)
Striatum			
4 weeks after infusion			
Total	2.8 ± 0.4 (100)	6.1 ± 0.8** (100)	12.3 ± 3.5** (100)
Neurons	0 (0)	0 (0)	0 (0)
Astrocytes	0.2 ± 0.1 (6)	1.4 ± 0.4** (22)	4.5 ± 1.4** (39)
$BrdU^+$/satellite cells	1.1 ± 0.1 (40)	3.2 ± 0.5* (53)	5.2 ± 1.1* (46)
$BrdU^+$/sat./astrocytes	0.05 ± 0.03 (1.8)	0.6 ± 0.2* (10)	1.7 ± 0.7* (14)

The brain areas were selected for unbiased quantification, as shown in Figure 1. Densities of newborn cells after aCSF, FGF-2, and EGF infusion are presented as the mean number of BrdU-positive cells (in thousands) per mm^3 ± SEM. *$p < 0.05$; **$p < 0.01$. To determine the cell type of BrdU-positive cells 4 weeks after infusion, we used NeuN as a marker for neurons and S100β for astrocytes. Percentages of cell types (numbers in parentheses) are based on the total density of BrdU-positive cells.

istry. As depicted in Figure 1*C*, two predetermined areas (50 × 50 μm) in the RMS and four areas (100 × 100 μm) in the granule cell layer (GCL) of the OB were analyzed on each section. All BrdU-positive nuclei in these selected areas were counted and presented as the number of cells (in thousands)/mm^3 (Table 1).

Dentate gyrus. Every 12th section (40 μm) from a coronal series was selected from each animal and processed for immunoperoxidase. Six sections from the dorsal hippocampus (AP +5.86 to +2.96 mm) were analyzed entirely for BrdU-positive cells in the molecular layer, the GCL, and the hilus. The subgranular zone, defined as a two-cell body-wide zone along the border of the GCL and the hilus, always was combined with the GCL for quantification. All BrdU-positive nuclei in these selected areas were counted and presented as cells (in thousands)/mm^3 (Table 2).

Cerebellum. On two sagittal sections of the cerebellum the fourth lobulus was analyzed entirely for the density of BrdU-positive cells in the molecular layer, GCL, and white matter. Cell numbers are expressed as BrdU-positive cells (in thousands)/mm^3 (Table 2).

Statistical analysis was performed with one-way ANOVA, followed by *post hoc* comparison with the Tukey *post hoc* test.

RESULTS

To study the effect of growth factors on the proliferation of adult neural progenitor cells *in vivo*, we chronically infused EGF, FGF-2, or aCSF for 2 weeks into the lateral ventricle of adult rats, using osmotic minipumps. BrdU was administered intraperitoneally during the period of growth factor infusion to label dividing cells. Animals either were killed on the last day of growth factor infusion to analyze the mitotic effect of the growth factors or were kept for an additional 4 weeks after terminating growth factor infusion to study the differentiation of the newborn cells. Both systems of adult neurogenesis, the SVZ/OB and dentate gyrus, were analyzed for BrdU-positive cells. The cerebellum served as a control area in the adult rat brain because it lacks detectable neurogenesis. To quantify the proliferation of neural progenitor cells and the survival of newborn cells after growth factor infusion, we determined the density of BrdU-positive cells by stereological counting techniques. To characterize cell fate, we combined BrdU labeling with the astroglial marker S100β, which labels astrocytic cell bodies (Boyes et al., 1986), and the neuronal marker NeuN, which recognizes neuronal cell bodies and nuclei (Mullen et al., 1992). The percentage of BrdU-positive cells colabeled either with NeuN or S100β was determined by triple immunofluorescence and confocal laser scanning microscopy and was multiplied by the overall density of BrdU-positive cells to determine the density of newborn neurons and astrocytes.

EGF effects on proliferation and differentiation of neural progenitors

Infusion of EGF induced a striking proliferation of the SVZ precursor population. Expansion of BrdU-positive cells was most pronounced in the lateral wall of the lateral ventricle (Fig. 2*C*). In addition, newborn cells were found in the medial and posterior circumference of the lateral ventricle, suggesting that progenitors also were recruited to divide in "quiescent" areas of the SVZ. Infusion of EGF resulted in "polyp-like" hyperplasias of the ventricle wall, which consisted of BrdU-positive cells that were immunonegative for either S100β or NeuN (Fig. 3). These EGF-induced hyperplasias had regressed completely after 4 weeks (Fig. 3*C*).

Quantification of the SVZ revealed a ninefold increase in the density of newborn cells over aCSF controls immediately after EGF infusion. The number of labeled cells present after 4 weeks remained increased relative to controls (Fig. 2, Table 1). There was also an increase in the number of labeled cells observed in adjacent areas, particularly in the striatum (Fig. 4*C*, Table 1), but also in cortex and septum (Fig. 4*F,I*), where the majority of newborn cortical cells was detected around the cannula tract (Fig. 4*F*). Interestingly, in the striatum triple labeling of BrdU-positive EGF-generated striatal cells with BrdU, NeuN, and S100β re-

Table 2. Density and cell fate of newborn cells in the dentate gyrus and cerebellum

Area	aCSF	FGF-2	EGF
Dentate gyrus			
1 d after infusion			
Granule cell layer	5.60 ± 0.49	4.87 ± 1.06	7.77 ± 1.10
Hilus	1.09 ± 0.34	1.14 ± 0.22	2.06 ± 0.59
Molecular layer	0.66 ± 0.11	0.92 ± 0.17	3.69 ± 0.32**
4 weeks after infusion			
Granule cell layer			
Total	3.44 ± 0.56 (100)	3.25 ± 0.32 (100)	2.84 ± 0.89 (100)
Neurons	3.19 ± 0.52 (93)	2.91 ± 0.27 (90)	1.50 ± 0.52** (53)
Astrocytes	0.01 ± 0.01 (0.2)	0.01 ± 0.01 (0.2)	1.13 ± 0.35** (40)
Hilus	0.57 ± 0.17	0.67 ± 0.14	1.02 ± 0.22
Molecular layer	0.62 ± 0.07	0.70 ± 0.10	2.32 ± 0.47**
Cerebellum			
1 d after infusion			
Molecular layer	0.081 ± 0.019	0.160 ± 0.035	0.133 ± 0.027
Granule cell layer	0.065 ± 0.027	0.113 ± 0.007	0.065 ± 0.025
White matter	0.084 ± 0.041	0.050 ± 0.031	0.082 ± 0.018
4 weeks after infusion			
Molecular layer	0.069 ± 0.008	0.073 ± 0.016	0.094 ± 0.009
Granule cell layer	0.079 ± 0.007	0.084 ± 0.021	0.123 ± 0.017
White matter	0.079 ± 0.012	0.095 ± 0.028	0.152 ± 0.027

The brain areas were quantified by unbiased sampling methods. Densities of newborn cells after aCSF, FGF-2, and EGF infusion are presented as the mean number of BrdU-positive cells (in thousands) per mm^3 ± SEM. **$p < 0.01$. To determine the cell type of BrdU-positive cells in the dentate gyrus 4 weeks after infusion, we used NeuN as a marker for neurons and S100β for astrocytes. Percentages of cell types (numbers in parentheses) are based on the total density of BrdU-positive cells.

vealed no BrdU-labeled neurons. Although a large number of BrdU-positive nuclei (45%) were associated closely with neurons, three-dimensional confocal analysis revealed that the BrdU-positive nuclei belonged to a cell body located in a different focal plane (Fig. 5). These closely attached BrdU-positive cells frequently colabeled (up to 30%) with S100β, suggesting that these cells were satellite cells of glial origin. Because this finding stands in contrast to a previous report of EGF-induced neurogenesis in the adult mouse striatum (Craig et al., 1996), we reanalyzed the sections from the striatum and cerebral cortex of each EGF-treated animal (4 weeks after infusion) looking for cells that appeared to be double-labeled for BrdU and NeuN. Detailed confocal z-series analysis of 20 cells per animal revealed invariably that none of the BrdU-positive nuclei was contained within a NeuN-positive neuron. Thus, of a total of >2800 newborn cells scored, none were neurons.

Many of the cells born in the SVZ migrate along the RMS into the OB, where they differentiate into neurons (Corotto et al., 1993; Lois and Alvarez-Buylla, 1993). On their way to the OB the progenitor cells can undergo cell division as well as differentiation into neuroblasts that express early neuronal markers like TuJ1 (Bonfanti and Theodosis, 1994; Thomas et al., 1996). Four weeks after infusion no residual BrdU-positive cells could be detected in the RMS (Fig. 6*F*). Although more cells are born in the SVZ in response to EGF treatment, significantly fewer BrdU-positive cells (40% of aCSF control) are present in the RMS (Fig. 6*C*, Table 1). The number of newborn cells that reached the OB after 4 weeks of EGF infusion also was reduced significantly (40% of the aCSF control, Table 1). PSA-NCAM, the polysialylated form of the neural cell adhesion molecule, appears to be required for migration of neuronal precursors within the RMS (Ono et al., 1994; Hu et al., 1996). Although PSA-NCAM expression was not quantified, it was detected by immunofluorescence labeling in all experimental groups (Fig. 6*A–C*). Therefore, EGF-induced reduction of newborn SVZ cells in the RMS was not attributable to the absence of PSA-NCAM after growth factor treatment. Triple immunofluorescence showed that the population of newborn cells that reached the GCL of the OB in control animals consisted of ~96% neurons and <0.1% astrocytes, whereas EGF not only reduced the number of cells reaching the bulb but also shifted the ratio toward a glial lineage (72% neurons/14% astrocytes) (Fig. 7, Table 1). The absolute density of newborn glia increased from 160 cells/mm^3 in controls to 2260 cells/mm^3 (14-fold) after EGF treatment (Table 1). Therefore, the shift was not simply a relative increase in newborn glia because of a decrease in newborn neuronal cells but also indicated an increased *de novo* gliogenesis.

Within the hippocampus, cells born at the boundary between the hilus and GCL migrate into the GCL before differentiating into neurons. All three layers of the hippocampal dentate gyrus (molecular layer, GCL, and hilus) were analyzed for the density of newborn cells after EGF treatment. The molecular layer of EGF-treated animals demonstrated a significant increase in the number of BrdU-positive cells. The majority of these cells was found in the immediate vicinity of the wall of the third ventricle. In the hilus and GCL, where neurogenesis normally occurs, the number of newborn cells was not altered significantly at the end of EGF infusion or 4 weeks later (Table 2). In aCSF-treated animals 92% of the newborn cells differentiated into neurons and <1% into astrocytes. In contrast, EGF changed this ratio to 52% neurons and 39% astrocytes (Fig. 8, Table 2), inducing a shift toward the glial fate that was even more pronounced than in the OB. The absolute density of newborn glia increased from 10 cells/mm^3 in controls to 1130 cells/mm^3 (>100-fold) after EGF treatment (Table 1). Even more prominent than in the OB, the shift toward glial differentiation was attributable to an increased

de novo gliogenesis and not merely a relative increase because of a decrease in newborn neuronal cells.

FGF-2 effects on proliferation and differentiation of neural progenitors

The density of newborn cells in the SVZ was increased by FGF-2, although to a lesser extent than by EGF, and the density of newborn cells into the adjacent striatal parenchyma was increased over aCSF controls (Figs. 2*B*, 4*B*, Table 1). None of the BrdU-positive cells in the striatum of FGF-2-treated animals was double-labeled for NeuN, although some newborn cells in the striatum were juxtaposed closely to neuronal cell bodies, as seen in EGF animals. FGF-2 also decreased the number of BrdU-positive cells in the RMS at the end of infusion (Fig. 6*B*, Table 1). As with EGF infusion and in aCSF controls, no BrdU-positive cells were detectable in the RMS 4 weeks later (Fig. 6*D–F*). However, in contrast to the EGF animals, the number of BrdU-positive cells found in the GCL of the OB 4 weeks after FGF-2 infusion was increased significantly over controls (Table 1). This general increase in the density of newborn olfactory cells was accompanied by an increase of newborn neurons. The number of newborn glial cells was not altered significantly, although this was probably because of the infrequent detection of BrdU/S100β-positive cells and a resulting high variance (Table 1).

In contrast to the SVZ/OB system, the generation of newborn cells in the hippocampal dentate gyrus was not affected by FGF-2 treatment. The ratio between newborn neurons and astrocytes also was not altered 4 weeks after FGF-2 treatment, indicating that both proliferation and differentiation of hippocampal progenitors were unaffected by the growth factor (Fig. 8, Table 2).

Analysis of newborn cells in the cerebellum revealed no significant changes at the end of growth factor treatment or 4 weeks after withdrawal of either growth factor (Table 2), indicating that this brain structure, which normally shows no adult neurogenesis, is unresponsive to these growth factors.

DISCUSSION

During development, growth factors provide important extracellular signals for regulating the proliferation and fate determination of stem and progenitor cells in the CNS (Calof, 1995). By infusing EGF and FGF-2 into the lateral ventricle of adult rats, we could show that the two populations of progenitors that continue to divide in the adult brain respond differently to these growth factors *in vivo*. Proliferation of hippocampal progenitors was unaffected by either EGF or FGF-2. In contrast, proliferation of subventricular progenitor cells increased after both FGF-2 and EGF administration, with EGF having a more dramatic effect. These findings are consistent with numerous *in vitro* studies that have shown that both factors can maintain responsive neural progenitors in cell cycle, thus expanding the progenitor population and delaying differentiation (Richards et al., 1992; Vescovi et al., 1993; Morshead et al., 1994; Sensenbrenner et al., 1994; Bouvier and Mytilineou, 1995; Gage et al., 1995a; Gritti et al., 1995, 1996; Palmer et al., 1995; Santa-Olalla and Covarrubias, 1995).

FGF-2 had a strong mitotic effect on the SVZ progenitors *in vivo*, but the migration of newborn cells in the RMS was diminished during the infusion period. However, 4 weeks after FGF-2 infusion, a larger number of newly generated cells were detected in the OB, indicating an increased migration of SVZ progenitors after withdrawal of FGF-2. *In vitro* results suggest that FGF-2 has the potential to keep uncommitted progenitors in cell cycle and to delay differentiation (Vescovi et al., 1993; Bouvier and Mytilineou, 1995; Kilpatrick and Bartlett, 1995; Palmer et al., 1995; Gritti et al., 1996). The biphasic response of RMS cells to FGF-2 could be attributable to increased proliferation in the SVZ, which reduces progenitor cell migration through the stream. After FGF-2 withdrawal a larger number of cells would be released into the RMS, generating more newborn cells in the OB 4 weeks later. Because 96% of the newborn olfactory cells differentiated into neurons, we conclude that FGF-2 had a stimulatory effect on the generation of OB neurons. However, because a very low number of newborn glial cells were detected here, conclusions about FGF-2-induced changes of the glial cell population in the OB are not possible. EGF infusion also expanded the SVZ precursor population while decreasing the number of newborn cells in the RMS. However, in contrast to FGF-2, EGF withdrawal reduced neurogenesis in the OB, whereas the genesis of astrocytes was stimulated. Although olfactory neurogenesis involves separate areas for cell division (SVZ), migration (RMS), and differentiation (OB), recent studies have shown that cell division and neuronal commitment of progenitor cells can occur in the migratory stream (Bonfanti and Theodosis, 1994; Menezes et al., 1995; Lois et al., 1996; Thomas et al., 1996). Astrocytes in the RMS are typically neither proliferating nor participating in the migration (Lois and Alvarez-Buylla, 1994; Lois et al., 1996). We assume that EGF acts on proliferation primarily in the SVZ and on differentiation primarily in the OB but also is influencing cells in the RMS.

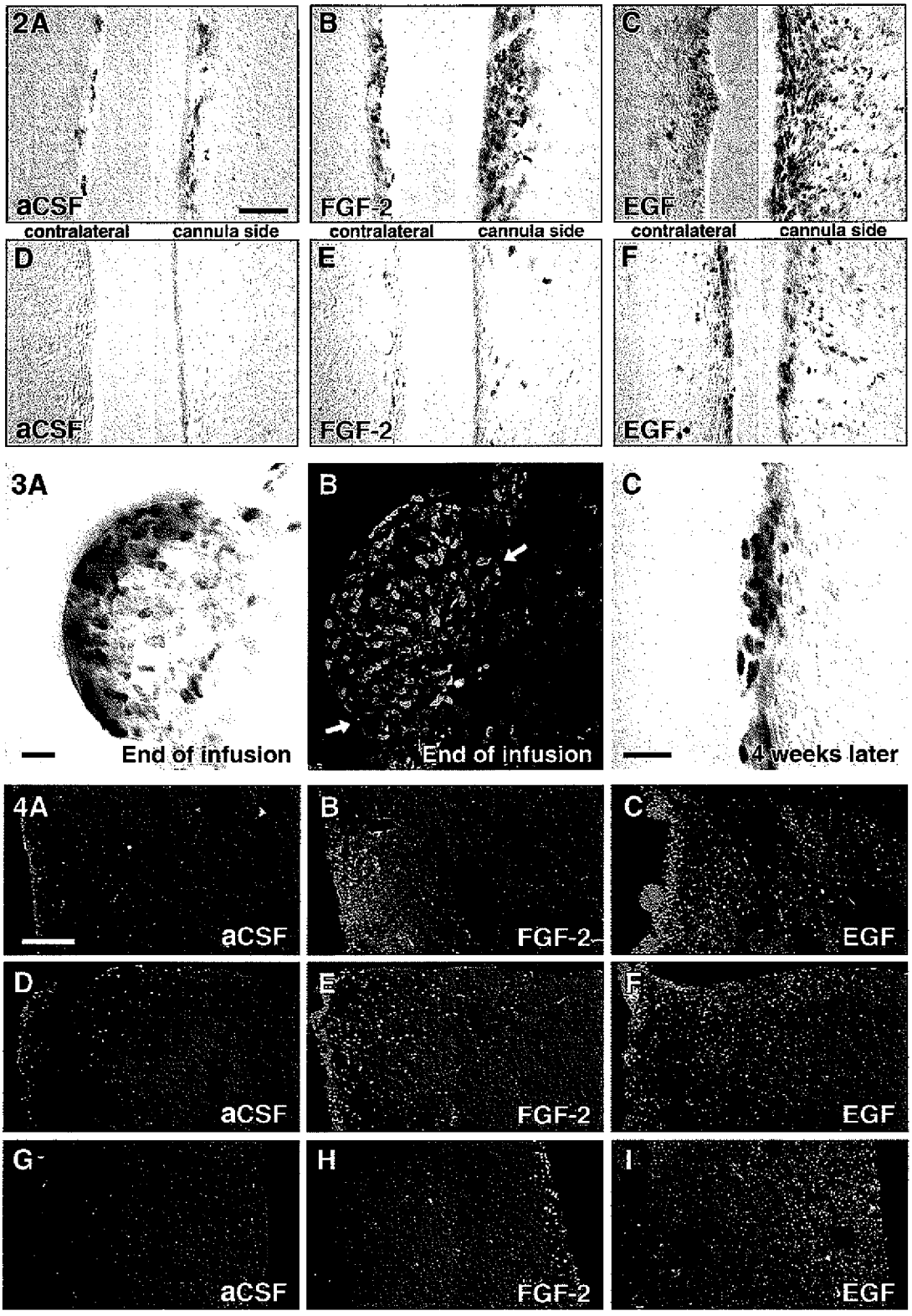
Progenitor populations in SVZ and hippocampus were affected differently by EGF. EGF had no proliferative effect on hippocampal progenitors, whereas even progenitors in quiescent areas of the SVZ, such as the medial and posterior regions of the ventricular wall, were recruited by EGF to enter the cell cycle. Normally,

→

Figure 2. BrdU-positive cells in the SVZ at the end of and 4 weeks after intracerebroventricular infusion of aCSF (*A*, *D*), FGF-2 (*B*, *E*), and EGF (*C*, *F*). Note the large expansion of the SVZ and the density of newborn cells in the striatum after FGF-2 administration (*B*), which are even more dramatic after EGF administration (*C*). Proliferation was more pronounced on the side of the cannula, as compared with the contralateral side. Four weeks after growth factor withdrawal, a high density of BrdU-positive cells was still present in the SVZ of EGF-treated animals (*F*). Scale bar in *A*, 50 μm.

Figure 3. "Polyp-like" hyperplasia in the SVZ of EGF-treated animals at the end of treatment (2 weeks). *A*, High density of BrdU-positive cells at the convex pole of a hyperplasia, which protrudes into the CSF-filled ventricle. *B*, BrdU-positive cells are immunonegative for neuronal (NeuN, *red*) and astrocytic markers (S100β, *blue*). The ependymal layer (S100β, *blue*) is discontinuous (*arrows*) in areas of growth. *C*, Density of BrdU-labeled cells is still increased; however, the hyperplastic changes completely regress 4 weeks after EGF withdrawal. Scale bars in *A*, *C*, 25 μm.

Figure 4. Increased number of BrdU-positive cells in the striatum (*A–C*), cortex (*D–F*), and medial septum (*G–I*) of EGF- and FGF-2-treated animals at the end of infusion. *D–F*, Note the increase of BrdU-positive cells along the cannula tract in the cerebral cortex (on the *left* side of the images). Shown are confocal microscopic images with immunofluorescent triple labeling for BrdU (*green*), NeuN (*red*), and S100β (*blue*). Scale bar in *A*, 200 μm.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EGF
3A
End of infusion
B
End of infusion
C
4 weeks later
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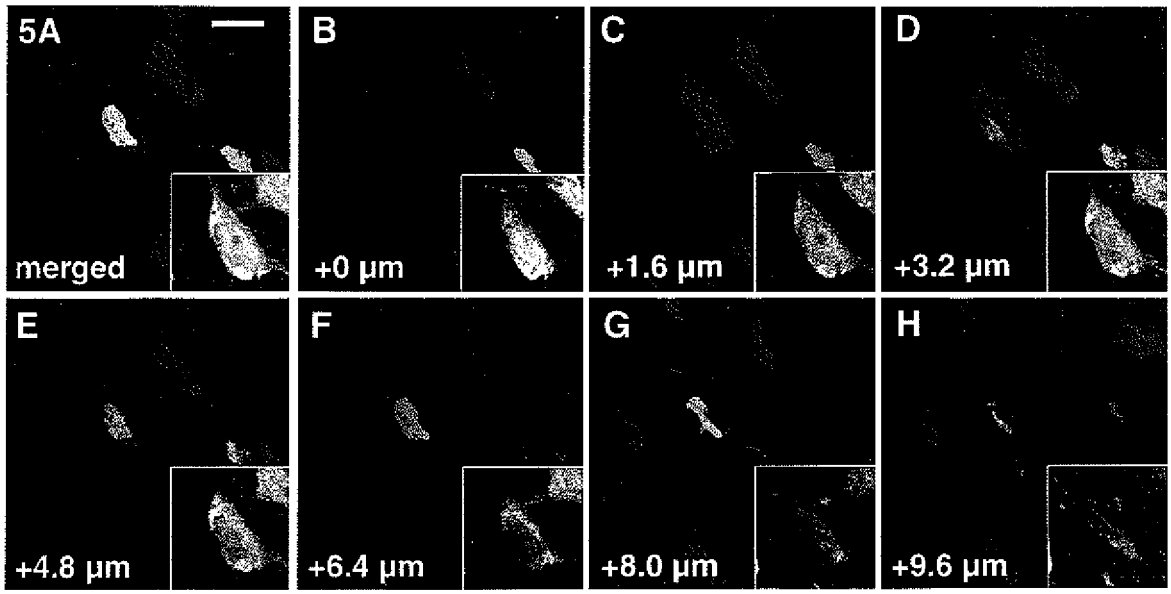
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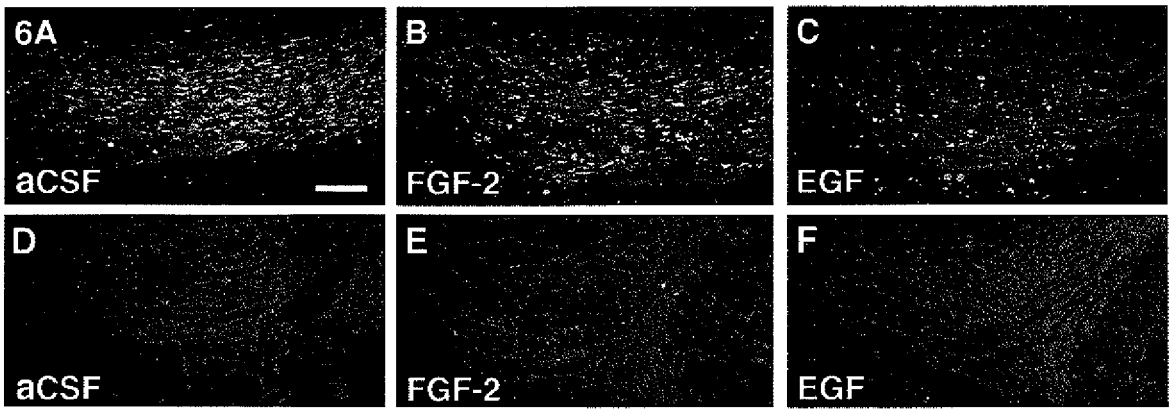
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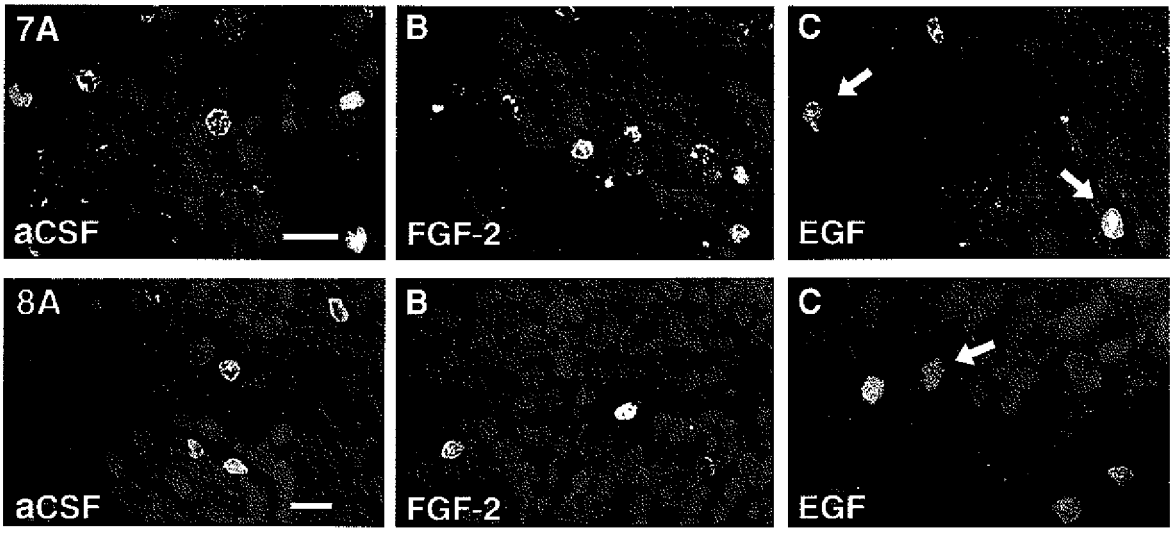
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the majority of precursor cells from the SVZ and hippocampus differentiates into neurons in their appropriate target regions (Kaplan and Hinds, 1977; Bayer, 1983; Cameron et al., 1993). However, in animals treated with EGF, the ratio of newborn neurons to astrocytes was altered, favoring glial differentiation. Among others, three alternative underlying cellular mechanisms are possible. (1) EGF could have opposite effects on separate glial and neuronal precursor populations, thus inducing proliferation of glial and reducing proliferation of neuronal progenitors. (2) Another explanation involves the effect of cell death on changes in progenitor populations. Developmental studies have shown direct evidence (Gould et al., 1991; Naruse and Keino, 1995; Blaschke et al., 1996) and studies of adult neurogenesis have shown indirect evidence (Morshead and van der Kooy, 1992) that naturally occurring cell death might play an important role in controlling the number of neuronal progenitors from SVZ and hippocampus. Therefore, general stimulation of proliferation by EGF in combination with increased cell death of neuronal progenitors could produce an increase in newborn glial cells without having a specific stimulatory effect of glial progenitors. The data from SVZ/OB could be interpreted in this way, because the increase in SVZ proliferation is equivalent to the increase in newborn OB glia. However, in the hippocampus the >100-fold increase in gliogenesis is not matched by a significantly higher proliferation. (3) The recent finding that multipotent neural stem cells exist in the adult rodent brain (Kilpatrick and Bartlett, 1993; Lois and Alvarez-Buylla, 1993; Morshead et al., 1994; Gage et al., 1995b; Palmer et al., 1995; Gritti et al., 1996; Reynolds and Weiss, 1996; Svendsen et al., 1996) suggests that EGF infusion could stimulate proliferation of stem cells in the brain but also could influence the fate of these multipotent cells toward a glial lineage.

The limited effect of FGF-2 on hippocampal progenitors *in vivo* contrasts with previous reports of the ability of FGF to maintain proliferative hippocampal progenitors *in vitro* (Ray et al., 1993; Gage et al., 1995a). It may be possible that hippocampal progenitors in their natural environment are not responsive to exogenous FGF-2 in the dose provided in this study. Alternatively, low penetration efficiency might reduce the availability of FGF-2 in the brain parenchyma (Gonzalez et al., 1994). Improved penetration could be achieved by addition of soluble FGF-binding molecules, such as heparan sulfate proteoglycans, to the infusion solution to prevent rapid absorption of FGF-2 by extracellular matrix molecules during infusion (Rapraeger et al., 1994).

Our findings are in part consistent with and in part in contrast to a recent study in adult mice (Craig et al., 1996). As in our study, EGF induced an expansion of the SVZ and an increased density of newborn cells into the adjacent striatum, cortex, and septum (Fig. 4). Whether the newborn cells migrated into these areas or were stimulated locally cannot be decided from our data, because multiple BrdU injections prevent the exact determination of birth place and time for these cells. However, in contrast to our findings, immunofluorescent double labeling of striatal and cortical cells with NeuN and BrdU in the previous study in mice (Craig et al., 1996) had indicated that newborn cells showed a neuronal phenotype. In our study three-dimensional confocal analysis revealed that NeuN and BrdU invariably were detected in separate cells (Fig. 5). A portion of the BrdU-positive cells that were juxtaposed to the NeuN-immunoreactive neurons expressed S100β, indicating that they were of astrocytic origin. Perineuronal satellite cells were described as early as 1913 by Ramón y Cajal as being positioned closely to neuronal perikarya and being of astrocytic and oligodendrocytic origin (Penfield, 1932; Ludwin, 1979, 1984). However, not all of the closely juxtaposed cells were S100β-positive, so we cannot exclude the possibility that some of the "unclassified" cells are uncommitted progenitor cells, which may differentiate into neurons at a later time point. In summary, although some experimental conditions, such as continuous EGF infusion, daily EGF doses, and immunohistochemical markers (BrdU, NeuN, and S100β), were comparable between the two studies, species differences (rat vs mouse) and, in particular, different histological analyses may account for the discrepancies.

A surprising finding of chronic EGF stimulation was the induction of pronounced hyperplasias in the ventricular wall, which protruded into the CSF-filled space (Fig. 3). Although receptors for both EGF and FGF-2 are expressed by subependymal cells (Gonzalez et al., 1995; Weickert and Blum, 1995; Craig et al., 1996), only EGF induced this hyperplasia. Numerous studies have shown the involvement of the EGF receptor family in tumorogenesis of the CNS (for review, see Berger et al., 1992; Collins, 1995; von Deimling et al., 1995). Four weeks after treatment the EGF-induced hyperplasia regressed completely, indicating that the continuous presence of EGF was required for the abnormal growth.

←

Figure 5. Close association of neurons with newborn cells (satellite cells). *A*, A NeuN-positive neuron (*red* and *black/white inset*) appeared to be colabeled with BrdU in a merged image resembling a regular fluorescent microscope image. *B–H*, Z-series analysis revealed that the NeuN-positive neuronal cell body is situated in a different focal plane from the BrdU-positive nucleus. Note that the NeuN-positive nucleus with nucleolus is visible in *C* and the BrdU-positive nucleus in *E* and *F*. Scale bar in *A*, 10 μm.

Figure 6. Reduced rostral migration of BrdU-positive cells at the end of EGF and FGF-2 infusions. Compared with aCSF controls (*A*), the number of BrdU-positive cells (*green*) in the RMS is decreased in FGF-2 (*B*) and decreased further in EGF-treated animals (*C*). However, PSA-NCAM (*red*), which is required for migration of progenitors within the RMS, is present in all groups. No BrdU-positive cells can be found at 4 weeks after infusion (*D–F*). Images *D–F* are immunofluorescent double labelings for BrdU (*green*) and S100β (*blue*). Note that scanning parameters for the confocal microscope are identical for *A–F*. Scale bar in *A*, 50 μm.

Figure 7. Cellular phenotype of newborn cells in the olfactory bulb. Cells in the olfactory granule cell layer were characterized at 4 weeks after infusion of aCSF (*A*), FGF-2 (*B*), or EGF (*C*) for BrdU (*green*), NeuN (*red*), and S100β (*blue*). After aCSF or FGF-2 treatment the vast majority of newborn cells double labels for NeuN (*green/red*). Note the reduced number of BrdU-positive cells that reach the olfactory bulb in EGF-treated animals (*C*; see also Table 1). The differentiation of EGF-induced progenitors was shifted toward a glial lineage. *Arrows* indicate newborn astrocytes (*green/blue*). Scale bar in *A*, 20 μm.

Figure 8. Cellular phenotype of newborn cells in the dentate gyrus. Cells in the hippocampal granule cell layer were characterized at 4 weeks after infusion of aCSF (*A*), FGF-2 (*B*), or EGF (*C*) for BrdU (*green*), NeuN (*red*), and S100β (*blue*). After aCSF or FGF-2 treatment the vast majority of newborn cells double labels for NeuN (*green/red*). The differentiation of EGF-induced progenitors has shifted toward a glial lineage (*C*). *Arrow* indicates a newborn astrocyte (*green/blue*). Scale bar in *A*, 20 μm.

In vitro models have been excellent tools for analyzing signals that influence the proliferation and fate of neural progenitor cells, but it has been difficult to determine how well these *in vitro* observations relate to signaling *in vivo*. By testing mitogens known to be effective *in vitro*, we have been able to show that progenitor populations in the adult rodent brain respond, in part, differently from *in vitro*. The site-specific responsiveness of progenitors to exogenous factors indicates that local cues play an important role in regulating neurogenesis *in vivo*. In the absence of *in vivo* signals, progenitors cultured in the presence of EGF proliferate and differentiate into neurons and glia, yet, *in vivo*, EGF has a stimulatory influence on proliferation and the genesis of glia but an unexpected limiting effect on the generation of neurons. This dichotomy emphasizes the importance of obtaining *in vivo* and *in vitro* results to identify more completely the factors that direct site-specific neuronal differentiation.

REFERENCES

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124:319–335.

Bayer SA (1983) ^{3}H-thymidine-radiographic studies of neurogenesis in the rat olfactory bulb. Exp Brain Res 50:329–340.

Berger F, Laine M, Hoffmann D, Verna JM, Charffanet M, Chauvin C, Rost N, Nissou MF, Benabid AL (1992) The EGF receptor pathway in human cerebral tumors. Neurochirurgie 38:257–266.

Blaschke AJ, Staley K, Chun J (1996) Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. Development 122:1165–1174.

Bonfanti L, Theodosis DT (1994) Expression of polysialylated neural cell adhesion molecule by proliferating cells in the subependymal layer of the adult rat, in its rostral extension, and in the olfactory bulb. Neuroscience 62:291–305.

Bouvier MM, Mytilineou C (1995) Basic fibroblast growth factor increases division and delays differentiation of dopamine precursors *in vitro*. J Neurosci 15:7141–7149.

Boyes BE, Kim SU, Lee V, Sung SC (1986) Immunohistochemical colocalization of S-100b and the glial fibrillary acidic protein in rat brain. Neuroscience 17:857–865.

Calof AL (1995) Intrinsic and extrinsic factors regulating vertebrate neurogenesis. Curr Opin Neurobiol 5:19–27.

Cameron HA, Woolley CS, McEwen BS, Gould E (1993) Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience 56:337–344.

Cattaneo E, McKay R (1991) Identifying and manipulating neuronal stem cells. Trends Neurosci 14:338–340.

Collins VP (1995) Gene amplification in human gliomas. Glia 15:289–296.

Corotto FS, Henegar JA, Maruniak JA (1993) Neurogenesis persists in the subependymal layer of the adult mouse brain. Neurosci Lett 149:111–114.

Craig CG, Tropepe V, Morshead CM, Reynolds BA, Weiss S, van der Kooy D (1996) *In vivo* growth factor expansion of endogenous subependymal neural precursor cell populations in the adult mouse brain. J Neurosci 16:2649–2658.

Gage FH (1994) Neuronal stem cells: their characterization and utilization. Neurobiol Aging 15(Suppl 2):S191.

Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, Peterson DA, Suhr ST, Ray J (1995a) Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. Proc Natl Acad Sci USA 92:11879–11883.

Gage FH, Ray J, Fisher LJ (1995b) Isolation, characterization, and use of stem cells from the CNS. Annu Rev Neurosci 18:159–192.

Goldman JE (1995) Lineage, migration, and fate determination of postnatal subventricular zone cells in the mammalian CNS. J Neurooncol 24:61–64.

Gonzalez AM, Carman LS, Ong M, Ray J, Gage FH, Shults CW, Baird A (1994) Storage, metabolism, and processing of ^{125}I-fibroblast growth factor-2 after intracerebral injection. Brain Res 665:285–292.

Gonzalez AM, Berry M, Maher PA, Logan A, Baird A (1995) A comprehensive analysis of the distribution of FGF-2 and FGFR1 in the rat brain. Brain Res 701:201–226.

Gould E, Woolley CS, McEwen BS (1991) Naturally occurring cell death in the developing dentate gyrus of the rat. J Comp Neurol 304:408–418.

Gritti A, Cova L, Parati EA, Galli R, Vescovi AL (1995) Basic fibroblast growth factor supports the proliferation of epidermal growth factor-generated neuronal precursor cells of the adult mouse CNS. Neurosci Lett 185:151–154.

Gritti A, Parati EA, Cova L, Frolichsthal P, Galli R, Wanke E, Faravelli L, Morassutti DJ, Roisen F, Nickel DD, Vescovi AL (1996) Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. J Neurosci 16:1091–1100.

Hauke C, Ackermann I, Korr H (1995) Cell proliferation in the subependymal layer of the adult mouse *in vivo* and *in vitro*. Cell Prolif 28:595–607.

Hu H, Tomasiewicz H, Magnuson T, Rutishauser U (1996) The role of polysialic acid in migration of olfactory bulb interneuron precursors in the subventricular zone. Neuron 16:735–743.

Kaplan MS, Bell DH (1983) Neuronal proliferation in the 9-month-old rodent—radioautographic study of granule cells in the hippocampus. Exp Brain Res 52:1–5.

Kaplan MS, Hinds JW (1977) Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science 197:1092–1094.

Kilpatrick TJ, Bartlett PF (1993) Cloning and growth of multipotential neural precursors: requirements for proliferation and differentiation. Neuron 10:255–265.

Kilpatrick TJ, Bartlett PF (1995) Cloned multipotential precursors from the mouse cerebrum require FGF-2, whereas glial restricted precursors are stimulated with either FGF-2 or EGF. J Neurosci 15:3653–3661.

Kuhn HG, Dickinson-Anson H, Gage FH (1996) Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci 16:2027–2033.

Levison SW, Goldman JE (1993) Both oligodendrocytes and astrocytes develop from progenitors in the subventricular zone of postnatal rat forebrain. Neuron 10:201–212.

Levison SW, Chuang C, Abramson BJ, Goldman JE (1993) The migrational patterns and developmental fates of glial precursors in the rat subventricular zone are temporally regulated. Development 119:611–622.

Lewis PD (1968) A quantitative study of cell proliferation in the subependymal layer of the adult rat brain. Exp Neurol 20:203–207.

Lois C, Alvarez-Buylla A (1993) Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci USA 90:2074–2077.

Lois C, Alvarez-Buylla A (1994) Long-distance neuronal migration in the adult mammalian brain. Science 264:1145–1148.

Lois C, Garcia-Verdugo JM, Alvarez-Buylla A (1996) Chain migration of neuronal precursors. Science 271:978–981.

Ludwin SK (1979) The perineuronal satellite oligodendrocyte. A role in remyelination. Acta Neuropathol (Berl) 47:49–53.

Ludwin SK (1984) The function of perineuronal satellite oligodendrocytes: an immunohistochemical study. Neuropathol Appl Neurobiol 10:143–149.

Luskin MB (1993) Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron 11:173–189.

Luskin MB, McDermott K (1994) Divergent lineages for oligodendrocytes and astrocytes originating in the neonatal forebrain subventricular zone. Glia 11:211–226.

Menezes JRL, Smith CM, Nelson KC, Luskin MB (1995) The division of neuronal progenitor cells during migration in the neonatal mammalian forebrain. Mol Cell Neurosci 6:496–508.

Michel RP, Cruz-Orive LM (1988) Application of the Cavalieri principle and vertical sections method to lung: estimation of volume and pleural surface area. J Microsc 150:117–136.

Morshead CM, van der Kooy D (1992) Postmitotic death is the fate of constitutively proliferating cells in the subependymal layer of the adult mouse brain. J Neurosci 12:249–256.

Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, Weiss S, van der Kooy D (1994) Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. Neuron 13:1071–1082.

Mullen RJ, Buck CR, Smith AM (1992) NeuN, a neuronal specific nuclear protein in vertebrates. Development 116:201–211.

Naruse I, Keino H (1995) Apoptosis in the developing CNS. Prog Neurobiol 47:135–155.

Ono K, Tomasiewicz H, Magnuson T, Rutishauser U (1994) N-CAM mutation inhibits tangential neuronal migration and is phenocopied by enzymatic removal of polysialic acid. Neuron 13:595–609.

Palmer TD, Ray J, Gage FH (1995) FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. Mol Cell Neurosci 6:474–486.

Paxinos G, Watson C (1986) The rat brain in stereotaxic coordinates. San Diego: Academic.

Penfield W (1932) Neuroglia and microglia, the interstitial tissue of the central nervous system. In: Special cytology (Cowdry EV, ed), pp 1147–1182. New York: Hoeber.

Privat A, Leblond CP (1972) The subependymal layer and neighboring region in the brain of the young rat. J Comp Neurol 146:277–302.

Rapraeger AC, Guimond S, Krufka A, Olwin BB (1994) Regulation by heparan sulfate in fibroblast growth factor signaling. Methods Enzymol 245:219–240.

Ray J, Peterson DA, Schinstine M, Gage FH (1993) Proliferation, differentiation, and long-term culture of primary hippocampal neurons. Proc Natl Acad Sci USA 90:3602–3606.

Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707–1710.

Reynolds BA, Weiss S (1996) Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 175:1–13.

Richards LJ, Kilpatrick TJ, Bartlett PF (1992) *De novo* generation of neuronal cells from the adult mouse brain. Proc Natl Acad Sci USA 89:8591–8595.

Santa-Olalla J, Covarrubias L (1995) Epidermal growth factor (EGF), transforming growth factor-alpha (TGF-alpha), and basic fibroblast growth factor (bFGF) differentially influence neural precursor cells of mouse embryonic mesencephalon. J Neurosci Res 42:172–183.

Sensenbrenner M, Deloulme JC, Gensburger C (1994) Proliferation of neuronal precursor cells from the central nervous system in culture. Rev Neurosci 5:43–53.

Sterio DC (1984) The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 134:127–136.

Suhonen JO, Peterson DA, Ray J, Gage FH (1996) Differentiation of adult hippocampus-derived progenitors into olfactory neurons *in vivo*. Nature 383:624–627.

Svendsen CN, Clarke DJ, Rosser AE, Dunnett SB (1996) Survival and differentiation of rat and human epidermal growth factor-responsive precursor cells following grafting into the lesioned adult nervous system. Exp Neurol 137:376–388.

Thomas LB, Gates MA, Steindler DA (1996) Young neurons from the adult subependymal zone proliferate and migrate along an astrocyte, extracellular matrix-rich pathway. Glia 17:1–14.

Vescovi AL, Reynolds BA, Fraser DD, Weiss S (1993) bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. Neuron 11:951–966.

von Deimling A, Louis DN, Wiestler OD (1995) Molecular pathways in the formation of gliomas. Glia 15:328–338.

Weickert CS, Blum M (1995) Striatal TGF-alpha: postnatal developmental expression and evidence for a role in the proliferation of subependymal cells. Brain Res Dev Brain Res 86:203–216.

EXHIBIT H

In vivo administration of EGF directly into the adult mouse subventricular zone has a greater effect on the frequency of neural progenitors than neural stem cells

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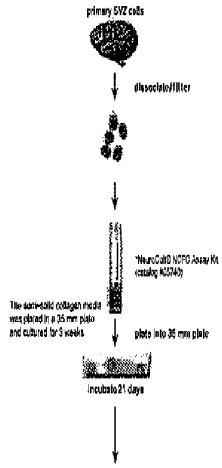
Introduction

A recently described single-step culture assay called the Neural Colony Forming Cell (NCFC) assay (Louis et al., 2004, abstract Soc. for Neuroscience) discriminates between neural progenitors and neural stem cells (NSC) thus providing a more accurate measure of NSC frequency than the Neurosphere Assay (NA). In the NCFC assay, colony size is an indication of proliferative potential and cells from colonies > 2 mm in diameter have the ability to self-renew over an extended period of time, generate large numbers of progeny and maintain multipotency. Hence the original cell that forms a colony > 2 mm in diameter is referred to as NCFC-NSC. Cells from colonies < 2 mm lack long-term self-renewal ability and are likely produced by a progenitor. The NCFC assay estimates the frequency of NCFC-NSC in the adult mouse subventricular zone (SVZ) to be 0.016 ± 0.01% (mean ± SE).

While NSCs are known to exist in the SVZ, the reports are controversial as to which of the different cell populations within this region, give rise to neurospheres and function as stem cells *in vitro*. We have previously reported that the Neurosphere Assay (NA), as it is currently used, is unable to discriminate between NSCs and progenitor cells and overestimates NSC frequency from adult brain by an order of magnitude (Adams et al., 2004 abstract Soc. for Neuroscience). Therefore, we have used the NCFC assay, to accurately measure NSC frequency and elucidate the effects of the *in vivo* infusions of EGF (Craig et al. 1996; Doetsch et al. 2002) and the anti-mitotic agent Ara-C (Doetsch et al, 1999) on NSCs in the adult SVZ region .

Methods

Figure 1. Neural Colony Forming Cell (NCFC) Assay procedure



After 21 days, colonies were enumerated and classified into one of four categories based on diameter:
A) >2.0 mm, B) 1-2 mm, C) 0.5-1 mm and D) < 0.5 mm.

Results

I. NCFC Assay

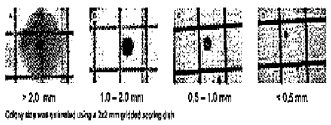


Figure 2. Colony size categories in the NCFC Assay.

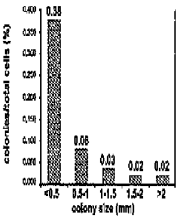


Figure 3. Frequency of NCFC-NSC in adult SVZ.

- The frequency of NCFC-NSC (generate colonies > 2 mm in diameter) is 0.016 ± 0.01% (mean ± SE; n=12)

Identification of neural stem and progenitor cells in a novel colony forming culture assay
Louis SA, Wagey R, Thomas TE, Eaves AC and Reynolds BA, 2004, Abstract Society for Neuroscience, 30th, Online

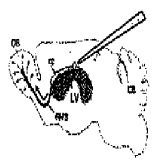
Cells from colonies > 2 mm in diameter are referred to as NCFC-NSCs. They have the ability to:
- self-renew over an extended period of time
- generate large numbers of progeny, and
- maintain multipotency

Colonies < 2 mm are produced by progenitors.

II. The Effects of EGF and Ara-C Infusion on Neural Stem and Progenitor Cells in the SVZ

It has been reported that the majority of stem cells isolated from the adult SVZ are derived from the rapidly dividing progenitor population and that EGF can convert these cells into stem cells *in vitro* (Doetsch et al. 2002), a conclusion supported in part by Gritti et al. 1999. These results are contrary to an earlier publication and not supported by the general view that stem cells are a rare population of relatively quiescent cells (Morshead et al, 1998, Morshead and van der Kooy, 2004). As the NA was used to measure NSCs in these studies, we suspect the controversial results may be due to the inability of the NA to distinguish between stem and progenitor cells thus leading to the misinterpretation of the assay.

To address the issue of stem cell kinetics within the SVZ and its response to EGF infusion the following experiments were performed:



(1) **7 day EGF ICV Infusion**
EGF was infused into the brain of adult mice for 7 days. After 7 days of infusion mice were sacrificed and the SVZ region dissected.

(2) **3 day Ara-C ICV Infusion**
Ara-C was infused into the brain of adult mice for 3 days. After 3 days of infusion mice were sacrificed and the SVZ region dissected.

We hypothesized that increasing (EGF infusion) or decreasing (Ara-C infusion) the number of proliferating cells within the SVZ would not effect the endogenous stem cell population.

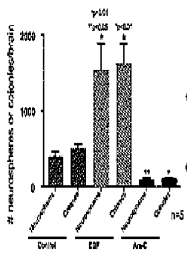


Figure 5. Number of neurospheres in NA and colonies in NCFC Assay.

- EGF infusion significantly increased the total number of neurospheres and colonies compared to control.
- Ara-C infusion significantly decreased the total number of neurospheres and colonies compared to control.

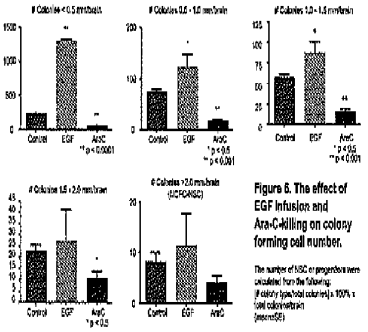


Figure 6. The effect of EGF infusion and Ara-C-killing on colony forming cell number.

The number of NSC or progenitors were calculated from the following:
(# colony type/total colonies) x 100% x total colonies/brain
(mean±SE)

- EGF significantly increased the number of colonies < 1.5 mm without affecting those > 2.0 mm in diameter (NCFC-NSC).
- Ara-C significantly reduced the numbers of colonies < 2.0 mm with no significant effect on those > 2.0 mm in diameter.

Summary

- EGF infusion into the lateral ventricle for 7 days increased the number of progenitor cells without significantly affecting the stem cell population.
- Ara-C infusion for 3 days resulted in a significant reduction in the number of progenitors in the SVZ without affecting the number of stem cells that could be isolated *in vitro*.

Conclusion

Our results support the hypothesis that the endogenous stem cell population within the adult SVZ is a relatively rare and quiescent population and that EGF does not convert progenitor cells into stem cells.

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EXHIBIT I

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of J.S. Reid et al.
Serial No: 09/129,028
Filed: August 4, 1998

Attorney Docket No. E8019-00003
Group Art Unit: 1649
Examiner: Chang Yu Wang

For: METHODS FOR TREATING NEUROLOGICAL DEFICITS

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1470
Alexandria, VA 22313-1470

DECLARATION OF JAMES H. FALLON

I, Dr. James H. Fallon, state the following:

1. I received my Ph.D. in anatomy, physiology and neuroscience in the Department of Anatomy at the University of Illinois, College of Medicine, and my postdoctoral training in chemical neuroanatomy in the Department of Neuroscience at the University of California, San Diego. I am currently Professor Emeritus of Anatomy and Neurobiology at the University of California, Irvine, and Professor of Psychiatry and Human Behavior where I have served as Chairman of the University faculty and Chair and President 0 the School of Medicine faculty. I am a Sloan Scholar, Senior Fulbright Fellow, and National Institutes of Health Research Career Awardee.

2. I am an inventor of Application Serial No. 09/129,028, entitled "METHODS FOR TREATING NEUROLOGICAL DEFICITS."

3. I have reviewed the office action dated April 15,2008, and U.S. Patent 5,980,885, to Weiss et al. (Weiss et al).

CERTIFICATE OF MAILING UNDER 37 C.F.R §1.8(a)

I hereby certify that this paper (along with any referred to as being attached or enclosed) is being

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☐ deposited with the United States Postal Service on May 5, 2009 with sufficient postage as EXPRESS MAIL LABEL NO in an envelope addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.	☒ filed electronically on May 5, 2009 with the United States Patent and Trademark Office.
Kathryn A. Touloumis (Signature of person mailing paper)	Kathryn A. Touloumis (Signature of person mailing paper)

4. I am aware that the examiner has rejected claims 4-5, 25-26, 40, 43-56 based on Weiss et al..

Overview of my research

5. My lab has studied various growth factors, including EGF, TGF-α, TGF-β, and FGF, and my research has focused on the TGF-α family of growth factors since 1982. My study of growth factors in the brain began with my discovery in 1984 that EGF was expressed in the mammalian brain – this was the first report that any growth factor was expressed in the brain, and that report was published in the journal *Science*. Fallon et al., "Epidermal growth factor immunoreactive material in the central nervous system: Location and development," Science 224: 1107-1109 (1984).

6. In 1993, based on my expertise working on a broad range of growth factors in the brain, I was asked to edit a book on neurotrophic factors (Loughlin, S.E., and Fallon, J.H. (1993) Neurotrophic Factors, Academic Press, N.Y.), and to write a chapter on the functional significance of the distribution of neurotrophic factors, which was published as Fallon, J.H., and Loughlin, S.E. (1993) The Functional Significance of the Distribution of Neurotrophic Factors. In: Neurotrophic Factors. (Loughlin, S.E., and Fallon, J.H., Eds) Academic Press, New York, pp.1-24.

7. My lab was also the first to show that administration of TGF-α in the presence of an injury signal can reverse loss of motor function in a neurological injury model. Fallon, et al., "In vivo induction of massive proliferation, directed migration, and differentiation of neural cells in the adult mammalian brain," Proc. Natl. Acad. Sci. 26(97): 14686-14691 (2000) (Fallon et al. 2000 (PNAS)).

8. In that work, we demonstrated that adult *in vivo* neurons can be generated at therapeutically effective levels high enough to repair neurological injury, even when administered weeks after the injury. Fallon et al. 2000 (PNAS). We also showed that TGF-α induces proliferation of adult CNS stem cells *in vivo,* and migration and differentiation of progenitor and progeny cells into various types of neurons in a brain with neurological injury or damage.

9. My PNAS reference was cited in a 2001 Stem Cell Report prepared by NIH for Congress, as the study showing proof of concept for adult stem cell treatment for neurological disorders. Report Prepared by the National Institutes of Health, "Stem Cells: Scientific Progress and Future Research Directions," June 2001, at page 84.

10. In addition to my work demonstrating recovery of function following intracranial administration of TGF-α, I showed that parenteral administration of TGF-α outside the CNS can also lead to functional recovery from stabilized injuries.

11. In our *in vivo* studies of TGF-α therapy, we used a rodent model that features a well defined neurological injury and measurable neurological deficits. By administering TGF-α under different protocols followed by functional and histological assessment, we were able to study whether therapeutic effects were obtained when using administration protocols for TGF-α. For example, see the protocols in the Specification, at pages 59, line 13 to page 60, line 25.

12. As discussed below, we found that intrastriatal administration of TGF-α or EGF in the absence of injury, does not give rise to the therapeutic levels of proliferation and migration observed when TGF-α is administered following injury. The increase in proliferation in neural cells when TGF-α is administered in the presence of an injury is shown in Figure 1 attached to my declaration. (See also Figure 1 in Fallon 2000 (PNAS) and the figure legend). Panel A shows the background number of stem and neuroprogenitor cells in the absence of TGF-α, while Panel B and C show an increased staining for stem and neuroprogenitor cells following TGF-α striatal infusions, and Panel C shows the proliferation and striatal ridge migration observed when TGF-α is administered intrastriatally following a neurological injury.

13. In addition, when we infused TGF-α into the striatum of the healthy rat brain, a low number of new cells were transiently observed in the subventricular zone immediately adjacent to the striatum, but then disappeared after the first week of infusion. This is illustrated in Figure 2, which shows the transient increase observed in the number of dividing cells observed in the subventricular zone (SVZ) following striatal TGF-α infusions.

14. However, when we injected TGF-α into rats suffering from a neurological injury, after several days, we observed significantly higher levels of proliferation. In addition, the mass of proliferating cells formed a striatal ridge and underwent a wave of migration to the damaged areas, where they differentiated into dopamine neurons. Fallon et al. 2000 (PNAS) at 14688. These results are also discussed, for example in the specification of the above-referenced application at page 19, lines 18-27 ("Intracerebroventricular (ICV) or intrastriatal infusions of TGFα or EGF without deafferentiation can induce proliferation, but degenerating, damaged (e.g., by deafferentiation or other injury), or otherwise abnormal (i.e., malfunctioning) cells must be present to facilitate migration, at least on a scale that is large enough to impact recovery from an associated neurological deficit").

15. Therefore, administration of TGF-α in the absence of injury does not induce sustained cell proliferation or migration of cells. In order to obtain massive cell proliferation into a striatal ridge and directed migration of the progenitor and progeny cells, injury or damage and a TGF infusion must be present

16. In addition, intrastriatal infusions of EGF did not induce formation of the striatal ridge, and did not give rise to the sustained proliferation and mass migration needed to obtain recovery from motor deficits. These results are discussed, for example, in the Specification at page 62, lines 19-30.

17. Intracerebroventricular administration of TGF-α or EGF also did not give rise to sustained proliferation or migration of progenitor and progeny cells needed to provide a therapeutic effect. This is discussed, for example in the Specification at page 67, lines 19-27 ("Intracerebroventricular (ICV) infusion of [TGF-α] ipsilateral to the lesion stimulated the buildup of cells in the adjacent ventricular wall, but did not induce formation of the striatal ridge in any of the animals.") and at page 72, line 18 to page 73, line 2, which discusses that intracerebroventricular (ICV) administration of EGF in the absence of injury, does NOT give rise to sustained proliferation or migration of progenitor and progeny cells needed to provide a therapeutic effect.

Differences between Weiss et al. and Reynolds et al. and the claimed invention

18. After reviewing Weiss et al., it is my understanding that what is described in those references differs from the claimed invention in several ways.

19. Weiss et al. does not describe any experiments administering any growth factor in vivo following injury or damage. Weiss did not carry out any actual experiments in an animal model for neurological injury.

20. In addition, Weiss et al. did not describe any actual experiments with any growth factor, where they determine that neurological deficits are present following a model injury; therefore, they could not establish whether any observed level of motor function was due to recovery, or whether the procedure to cause injury resulted in less severe injury.

21. Weiss et al. did not describe any experiments in which they administered TGF-α *in vivo*. Weiss et al. carried out their experiments using EGF in the presence or absence of FGF. Weiss et al., examples 27-30.

22. The actual *in vivo* experiments reported in Weiss et al. featured intracerebroventricular administration of EGF (in the presence or absence of FGF-2). Weiss et al. at Example 27.

23. Weiss et al. did not describe any experiments showing that administration of any growth factor induces migration of neural stem cells to site of injury or damage to replace lost cells or to obtain a therapeutic effect.

24. Weiss shows that the results observed using one growth factor do not necessarily provide an expectation that the same results will be obtained using other factors: For example, at column 47, lines 33-43, Weiss reports that administration of EGF, but not FGF-2, leads to (low levels of undirected) migration. Weiss et al. also report that the results in Table II exclude TGF-α from the list of tested factors, and further shows that various "growth" factors have various regulatory effects when used in combination with EGF and/or FGF.

Following the Method of Weiss et al. Would Not Give a Therapeutic Effect

25. I have conducted experiments that show that the administration of TGF-α does not always induce migration of cells to the site of neurological damage.

26. In order to determine, for example, whether different modes of administration of TGF-α would give rise to neurogenesis of functional neurons and therapeutic effects, including modes of administration based on the ICV administration methods in Weiss et al., experiments were carried out under my supervision in my laboratory at UCI.

27. As set forth in Weiss *et al.*, Examples 27-30, an infusion cannulae attached to a 0.5 ul/hour ALZET osmotic mini-pumps filled with 5, 20 or 50 μg/ml of EGF (or other growth factors) were surgically implanted into the lateral ventricle. The infusion pump cannula was secured to the mouse skull by use of an acrylic cement, and EGF was infused for 6 consecutive days, at the 0.5 ul/hour rate. Experiments were also conducted using TGF-α, EGF, FGF, BDNF, NGF, GDNF, NT3 and NT4, alone, or in combination in unlesioned animals.

28. In examples 27-30, Weiss et al. does not set forth the buffer used for the growth factor solution. Example 30 indicates that Weiss et al. used sterile saline + 0.1% albumin as a control buffer for in vivo infusion experiments. Experiments were carried out using growth factors in a physiological saline solution and in saline containing 0.1% bovine serum albumin.

29. Although it is not mentioned in Weiss et al., my lab also performed experiments using growth factors in artificial cerebrospinal fluid (a-CSF). As discussed, e.g., in example 2, beginning at page 45 of U.S. Serial No. 09/129,028, I also conducted experiments administering 50.0 μg/ml TGF-α in a-CSF outside the ventricles.

30. We monitored animals for 2 weeks or up to 8 weeks following administration of the growth factors.

31. In all of these experiments, my lab never obtained neurogenesis of functional neurons or therapeutic results following intracerebroventricular administration of any growth factor

(or combination of growth factors, including EGF + FGF), regardless of the concentration of the growth factor administered intracerebroventrically, whether saline or a-CSF was used as the buffer for the growth factor, or whether I waited 2 weeks or 8 weeks following administration of the growth factor. This includes experiments in which 50 μg/ml of EGF (with or without 50 μg/ml FGF-2) and TGF-α were administered intracerebroventrically for 6 days to lesioned (MCAO stroke model or 6-OHDA Parkinson's model), or unlesioned animals, animals were monitored for 2 to 8 weeks to obtain functional/behavioral data, and some animals were sacrificed at 2 to 8 weeks following growth factor administration, to obtain histological samples to determine whether sustained proliferation and directed migration had occurred.

32. In all experiments where the growth factor or factors were administered intracerebroventrically, I **never** observed any behavioral improvement and only very minimal, transient levels of cell proliferation were observed which didn't last more than 2 weeks. In the experiments conducted by following the example in Weiss *et al.*, I observed only very low levels of transient proliferation and undirected migration away from the ventricle wall, at the levels observed by Weiss et al. (that is, no more than a few hundred cells). I never observed mass, radial, directed migration of progenitor cells or progeny following intraventricular administration of any of TGF-α, EGF, FGF, BDNF, NGF, GDNF, NT3 and NT4, or EGF + FGF, in lesioned or unlesioned animals. I never observed any behavioral recovery in any animal treated with any growth factor in any buffer, or at any concentration, administered in the cerebral ventricles.

33. As discussed above, administration of TGF-α in the absence of injury does not induce sustained cell proliferation or migration of cells

34. In addition, as discussed above, ICV administration of TGF-α does not give the sustained cell proliferation or migration needed to induce migration of cells to the site of neurological damage to obtain a therapeutic effect.

35. Therefore, the methods according to the examples set forth in Weiss et al. did not work. The methods of Weiss et al. does not yield neurogenesis of functional neurons or therapeutic results.

36. There are several reasons why administration of EGF or TGF-α in the ventricles has only a minimal, transient effect on number of cells and causes low levels of undirected cell movement. First, growth factors administered in the ventricles are diluted by the cerebrospinal fluid.

37. Second, barriers to migration of new neural cells from the subventricular zone (SVZ) limit their migration path from the SVZ to the olfactory nerve, where they normally replenish the cells involved in the sense of smell. As a result any new cells in the SVZ cannot migrate to the site of injury. This is illustrated in Figures 3-9. Figure 3 shows the physical barriers to migration of cells out of the SVZ following administration of TGF-α in the ventricles. Figures 4 and 5 illustrate the normal migration route of progenitor cells from the lateral ventricle (LV) to the olfactory bulb (OB). Figures 6-9 show the concentration following administration of a factor into the ventricles, and the flow of CSF containing the factor out of the ventricles.

38. As another example, a single, bolus injection of TGF-α also does not give rise to sustained cell proliferation or migration. I have tested effects of a single bolus of TGF-α, and have observed that it does not give rise to sustained proliferation or migration of cells, or any therapeutic effect.

39. Following administration of EGF in the ventricles, Weiss observed only very small, transient increases in the number of cells and undirected migration of cells only over short distances—this minimal "proliferation" and "migration" was not sustained, and would not have been sufficient to obtain a therapeutic effect. In fact, procedures such as injections of buffer into the ventricles or striatum give rise to low levels of proliferating cells, as a transient, minimal response to the injury. As discussed above, and shown in Figure 2, these cells are only transiently observed and disappear within 10 days.

40. Based on the report by Weiss et al. that the maximum number of new cells observed was 350 cells per rat brain, Weiss et al. observed on average less than one cell per every 2 sections, based on the size of the rat caudate putamen and a reported section thickness of 30 uM (Weiss et al. at col. 47, lines 24-43).

41. In contrast, in my experiments administering TGF-α, I observed at least two orders of magnitude higher number of proliferated cells than was observed by Weiss et al. in their experiments administering EGF in the ventricles.

42. Normally, stem cells and new neurons are present in low numbers, but the number of new cells is dramatically increased in the presence of injury or damage when TGF-α is administered.

43. Therefore, even if one chose to administer TGF-c and used the ICV administration method of Weiss, no therapeutic effect would have been obtained.

44. Thus, administration of TGF-α into the ventricles did not give sustained proliferation or migration. Accordingly, Examples 27-30 of Weiss et al. are inoperable, even when one chooses independently to follow those methods using TGF-α.

Administration of TGF-α in the CNS Near the Site of Injury, Outside the Ventricles, Gives Rise to Significantly Greater Numbers of Proliferating Cells, Induces the Migration of Neural Stem Cells and Progeny to the Site of Injury, and Gives Rise to Therapeutic Effects

45. In contrast, using the methods of this invention, administration of TGF-α outside the ventricles in the presence of injury gives rise to proliferating cells at levels significantly higher than the number of new cells observed by Weiss et al. following administration of EGF, or the level of cells obtained following administration of TGF-α to the ventricles.

46. This is demonstrated in the above referenced application, and in Fallon et al. 2000 (PNAS).

47. In addition, my lab investigated whether peripheral, intranasal administration of TGF-α induced proliferation and migration of progenitor and progeny cells toward the site of injury in the adult rodent brain, when administered at least two months following injury from a transient middle cerebral artery occlusion (MCAO). By that time, the injury had completely stabilized and no further improvement of the injury symptoms were expected.

48. After four weeks of TGF-α infusions (2 months after the MCAO), a dramatic increase in the number of proliferating and migrating cells was observed, as demonstrated by the

incorporation of bromodeoxyuridine (BrdU), and the expression of the neural progenitor cells marker, nestin and striatal precursor Meis2 into the ipsilateral forebrain. These results demonstrate that TGFα, when administered following injury, induces the proliferation and mobilization, en masse, of thousands of precursor cells.

49. Figure 11 shows the histological results obtained when PGS and TGF-α are administered in the absence of injury. When TGF-α is administered n the absence of injury (panels C and D), the number of proliferating (panel C) and neuroprogenitor cells (panel D) is significantly lower than the number observed when TGF-α is administered in the presence of injury. Compare, for example, panel G in Figure 10 to panel C in Figure 11.

50. Figure 12 shows the results of behavioral studies to monitor functional recovery following the simulated stroke in rats. In the rats used in this study, behavioral data measuring motor activity showed significant improvement after infusions of TGFα.

51. Animals were assessed for motor function following simulated stroke, and for functional improvement resulting from TGFα infusions. Data from the cylinder test, which assesses forelimb asymmetry after simulated stroke, and is used measures chronic sensorimotor deficits, is shown in Figure 12. Asymmetric use of forelimbs (proportion of forelimb use differing from 0.5) reflects a sensorimotor deficit in the lesser used forelimb. The intact forelimb is used more after the stroke procedure, while non-impaired animals exhibit symmetric use of the forelimbs. The dark blue line (bottom line) shows the results for control animals (no MCAO procedure), who received PBS. The light blue line (second from the bottom) shows the results for control animals receiving TGF-α. The red line (third from the bottom) shows the results for injured animals receiving TGF-α. The change from around 0.5 at time zero to over 0.8 at around 35 days reflects a motor deficit, and the subsequent decrease back to around 0.6 reflects a therapeutic effect and recovery of some motor function. The yellow line (top line) shows the results for injured animals receiving PBS. The value changes from around 0.5 at day zero, to close to 1.0 at day 35, with a decrease back to around 0.8 at day 65. These results show a significantly greater recovery of motor function by injured rats subjects receiving TGF-α.

52. This study demonstrates that the methods of this invention can be used to administer TGFα intranasally to obtain a statistically significant difference in the proliferation, and differentiation of endogenous, adult stem cells for treatment of chronic stroke. In the rats used in this study, both histological measurement of new neural cells and behavioral data measuring motor activity showed significant improvement after infusions of TGFα.

The undersigned Declarant declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

March 4, 2009

Dr. James H. Fallon

FIGURE 1

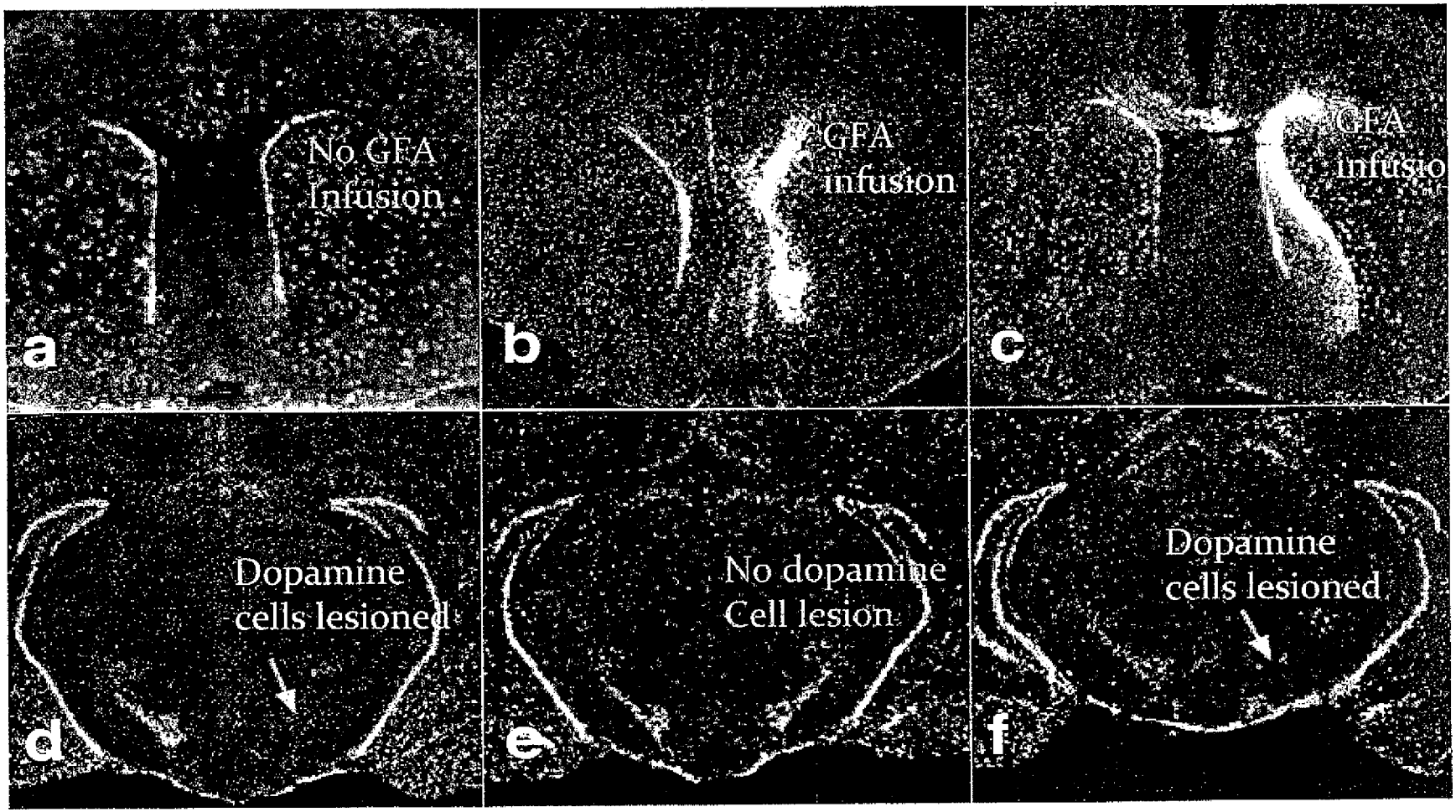
No GFA
Infusion
a
GFA
infusion
b
GFA
infusio
c
Dopamine
cells lesioned
d
No dopamine
Cell lesion
e
Dopamine
cells lesioned
f

FIGURE 2

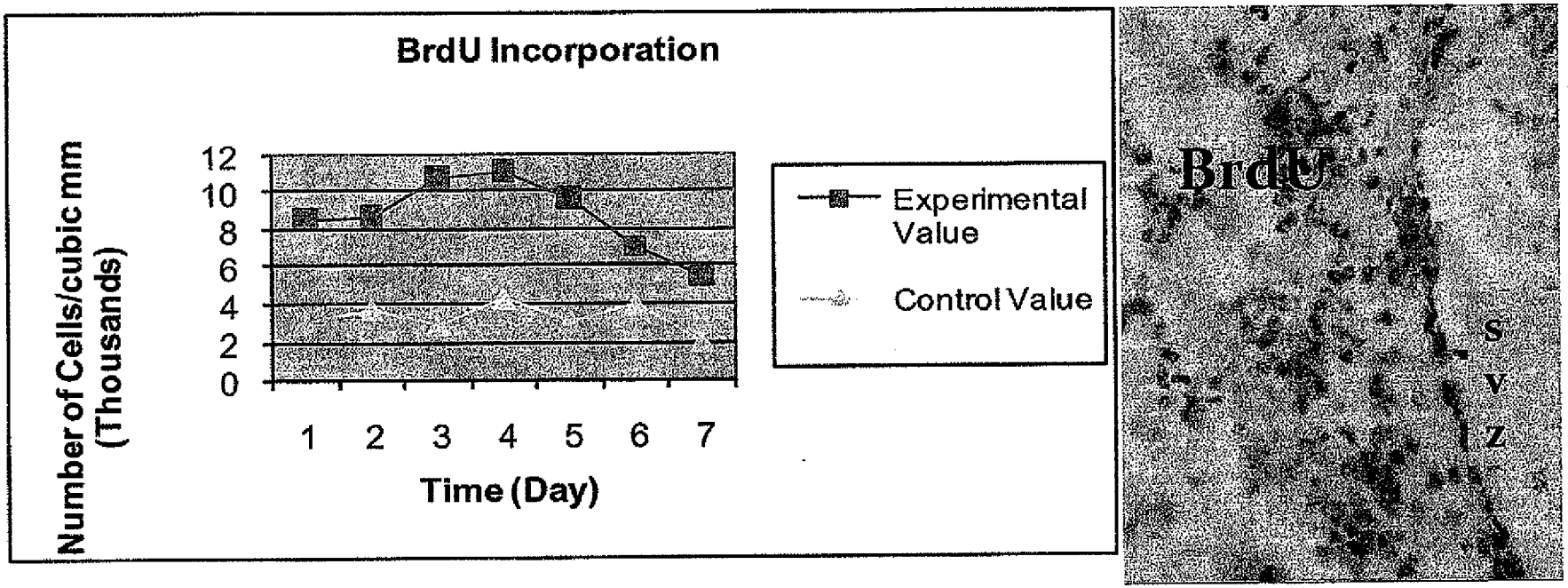
BrdU Incorporation
Number of Cells/cubic mm (Thousands)
12
10
8
6
4
2
0
1 2 3 4 5 6 7
Time (Day)
Experimental Value
Control Value
BrdU
S
V
Z

FIGURE 3: Barriers to migration of cells following administration in the ventricles

SLIT

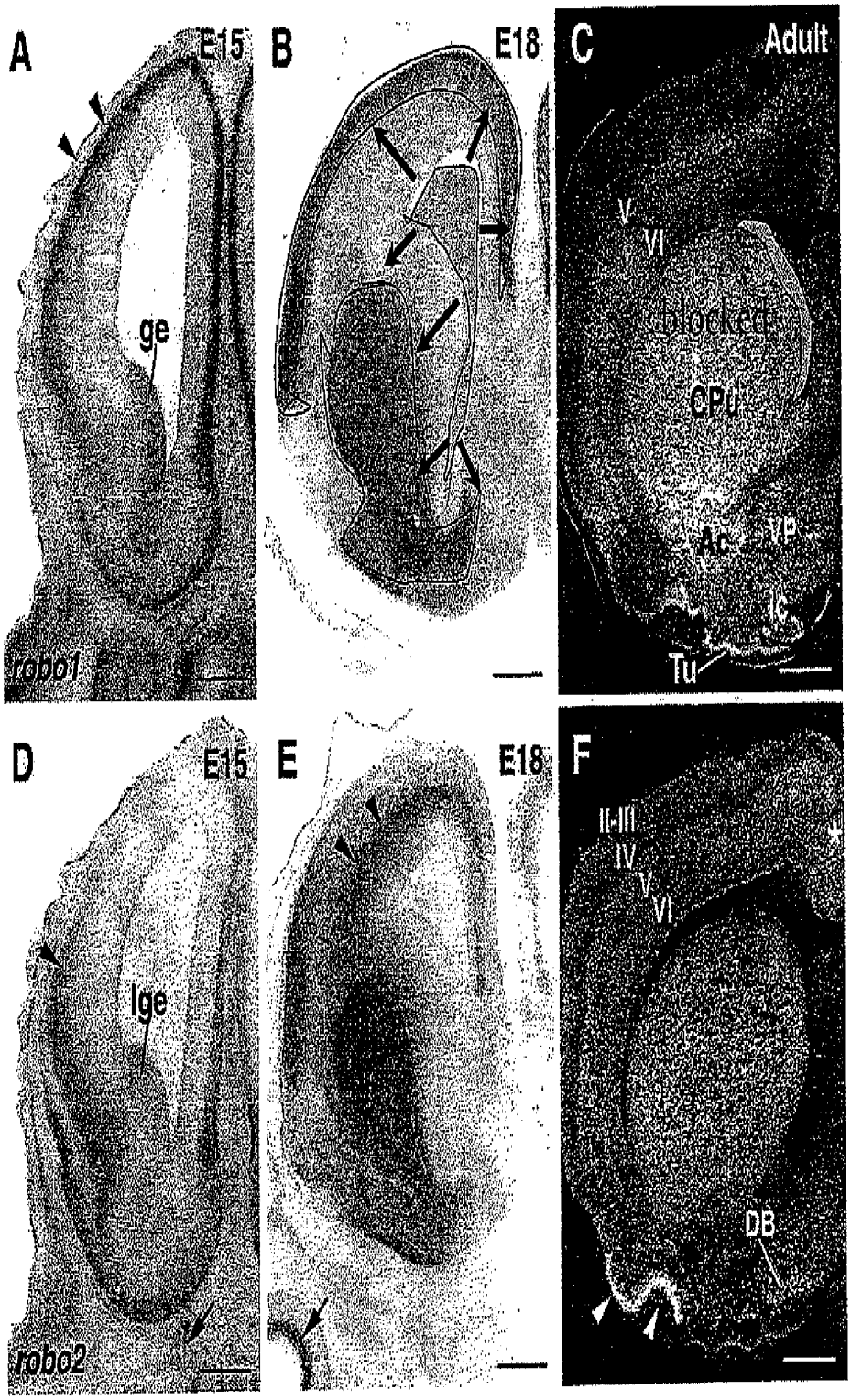

FIGURE 4: Barriers to migration of cells

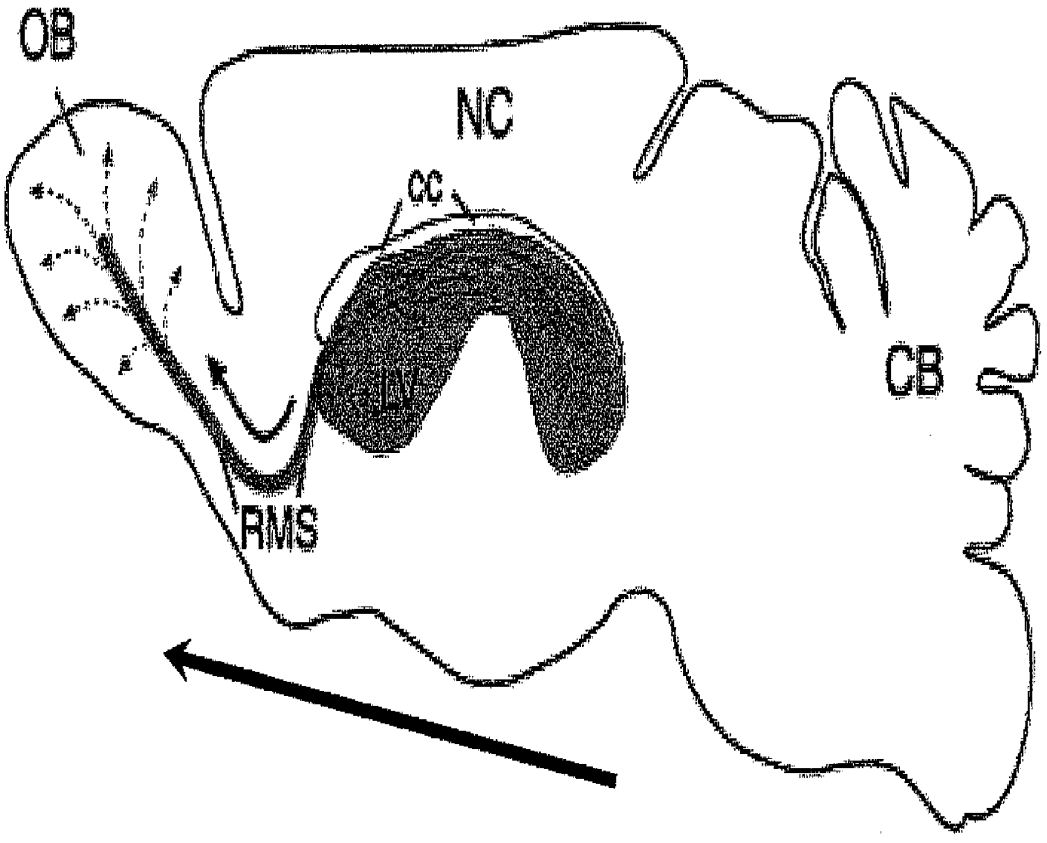

FIGURE 5: Barriers to migration of cells

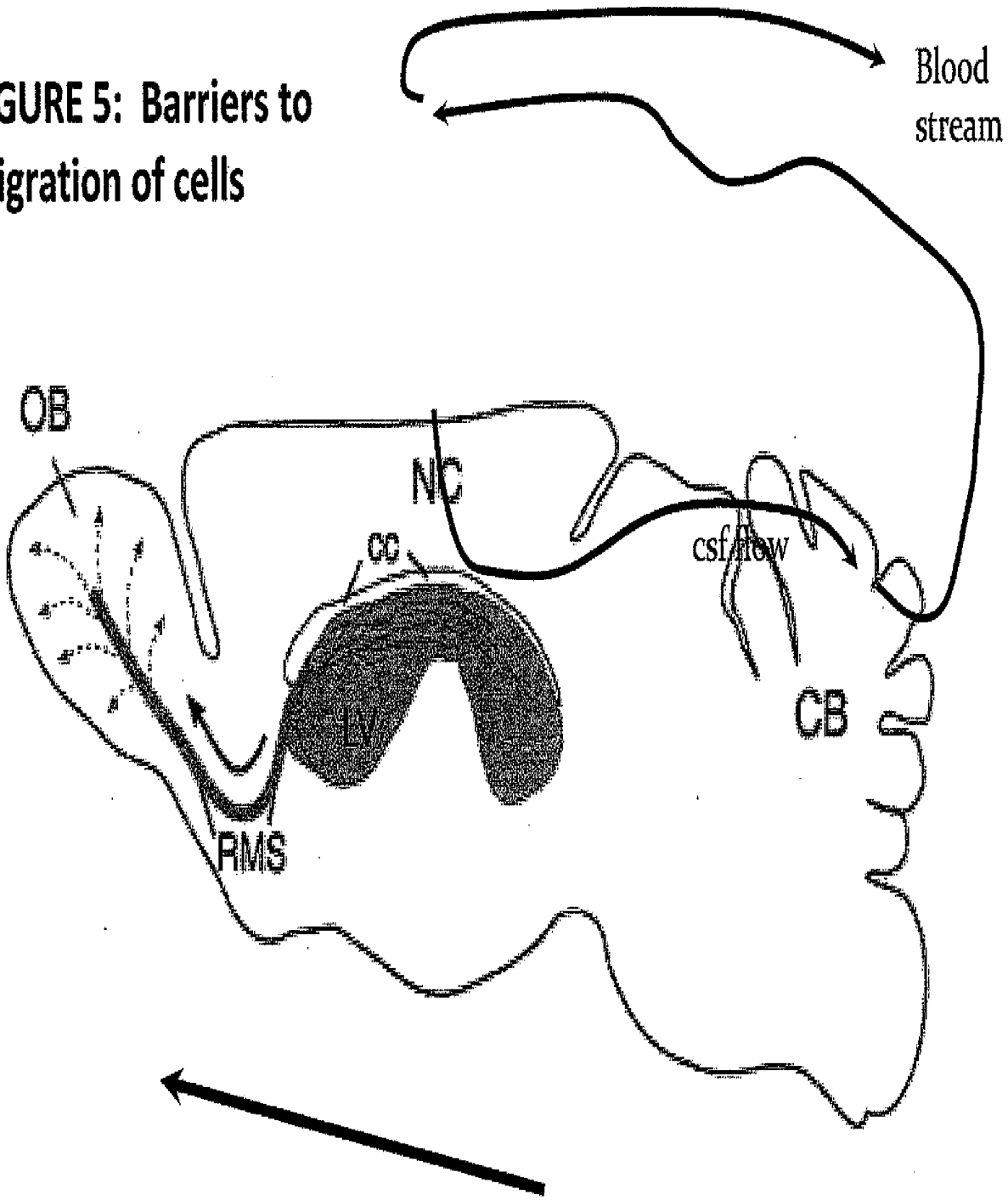

FIGURE 6 CSF FLOW FROM VENTRICLES

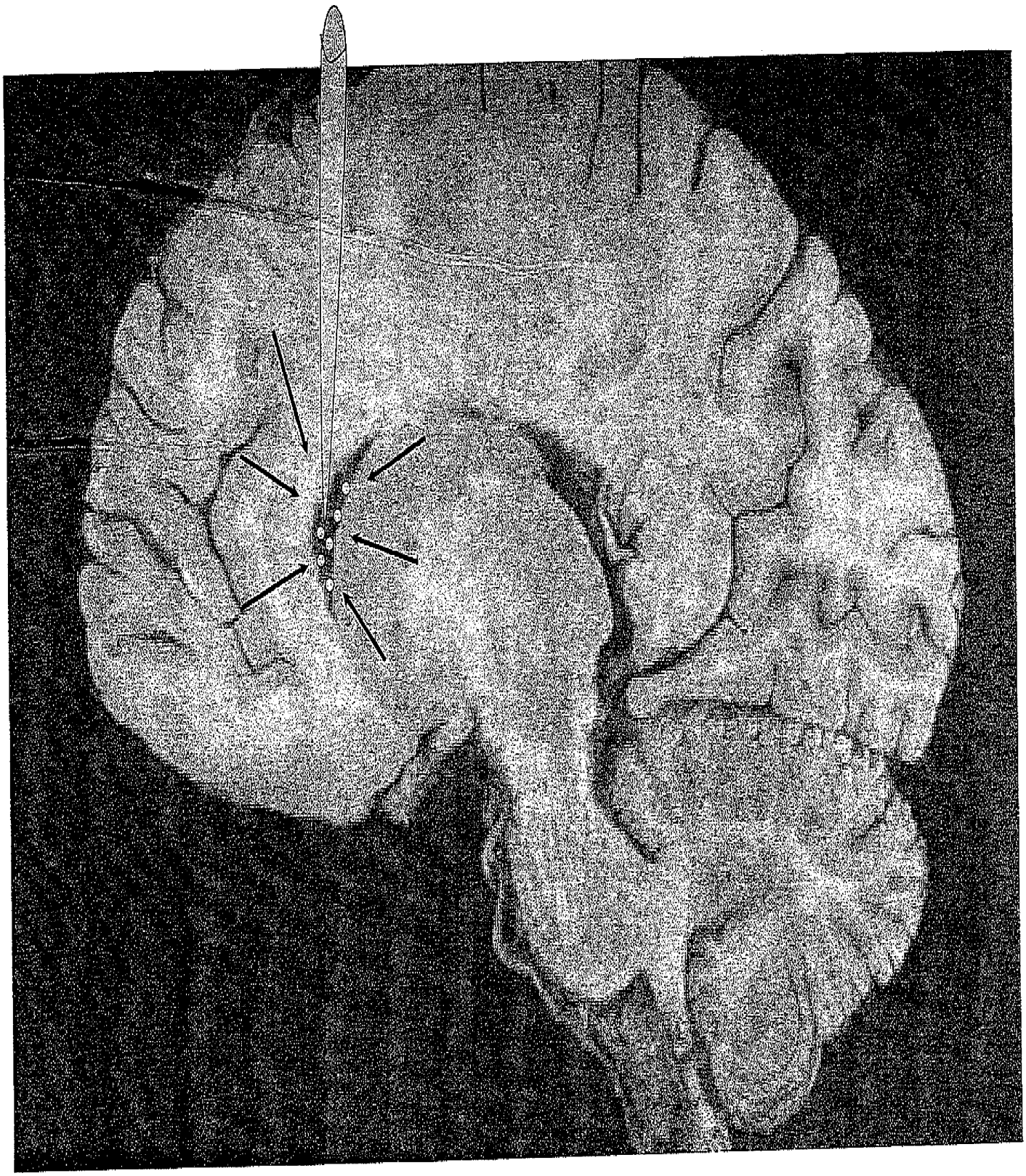

FIGURE 7: CSF FLOW FROM VENTRICLES

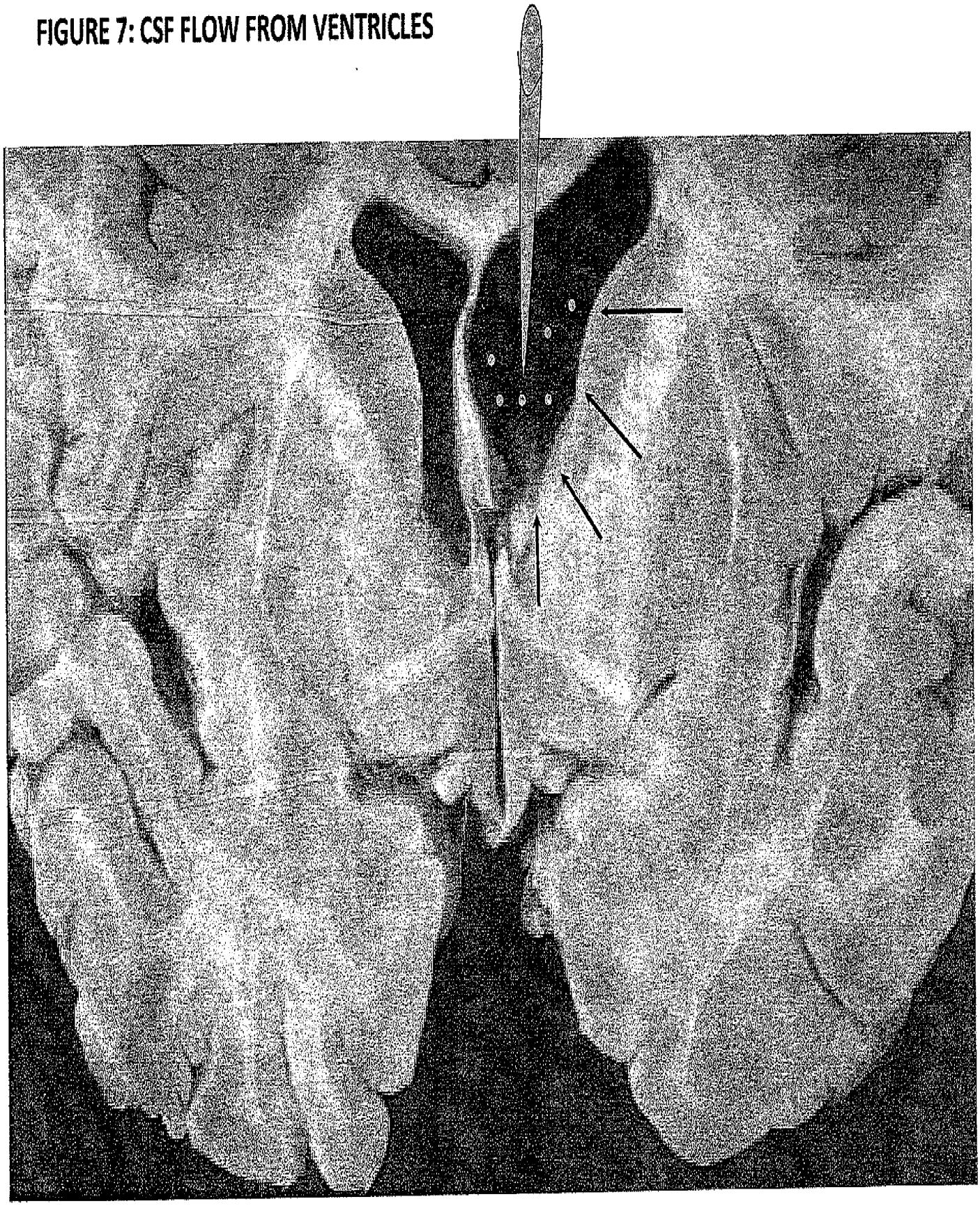

FIGURE 8: CSF FLOW FROM VENTRICLES

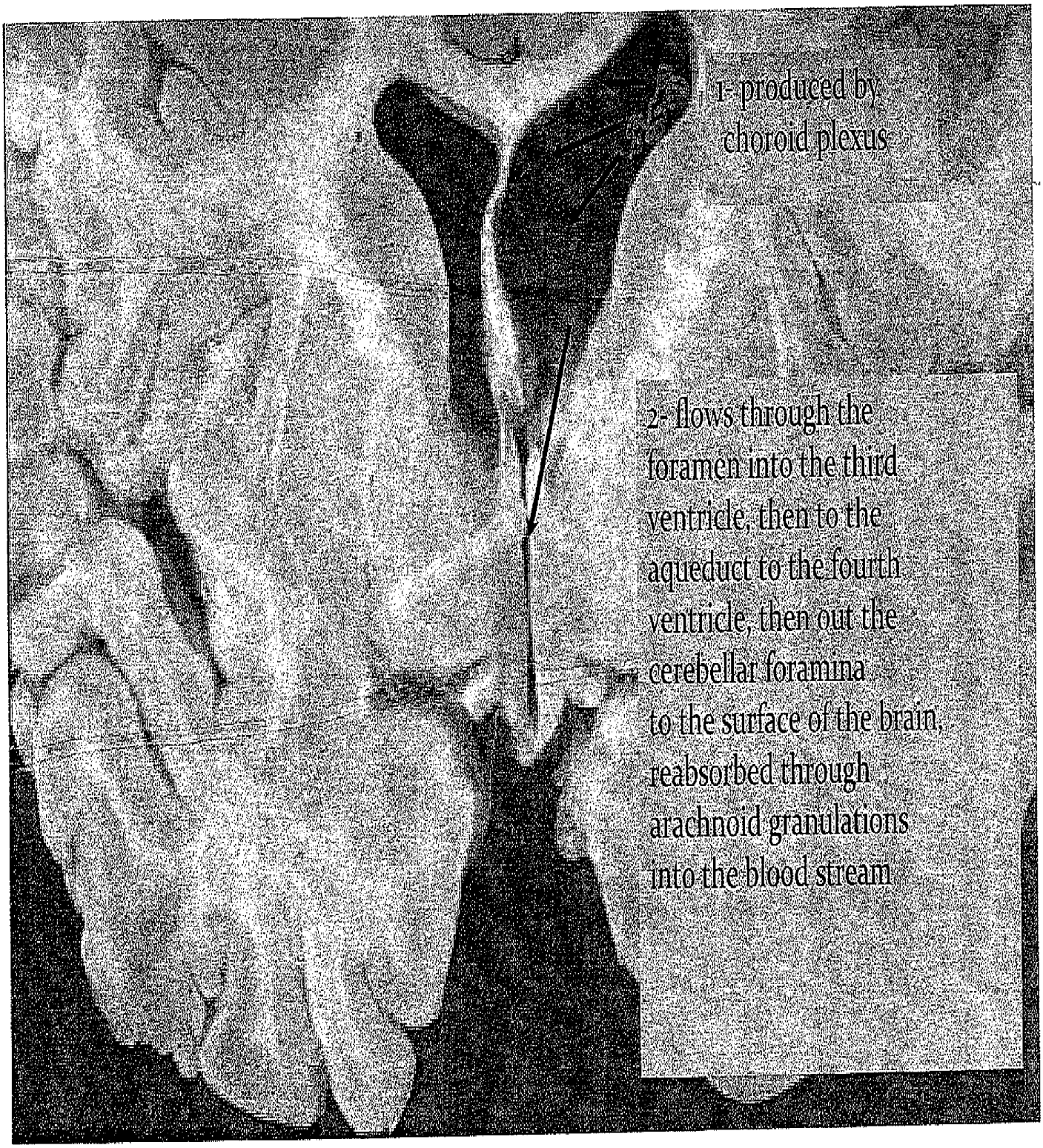

FIGURE 9: CSF FLOW FROM VENTRICLES

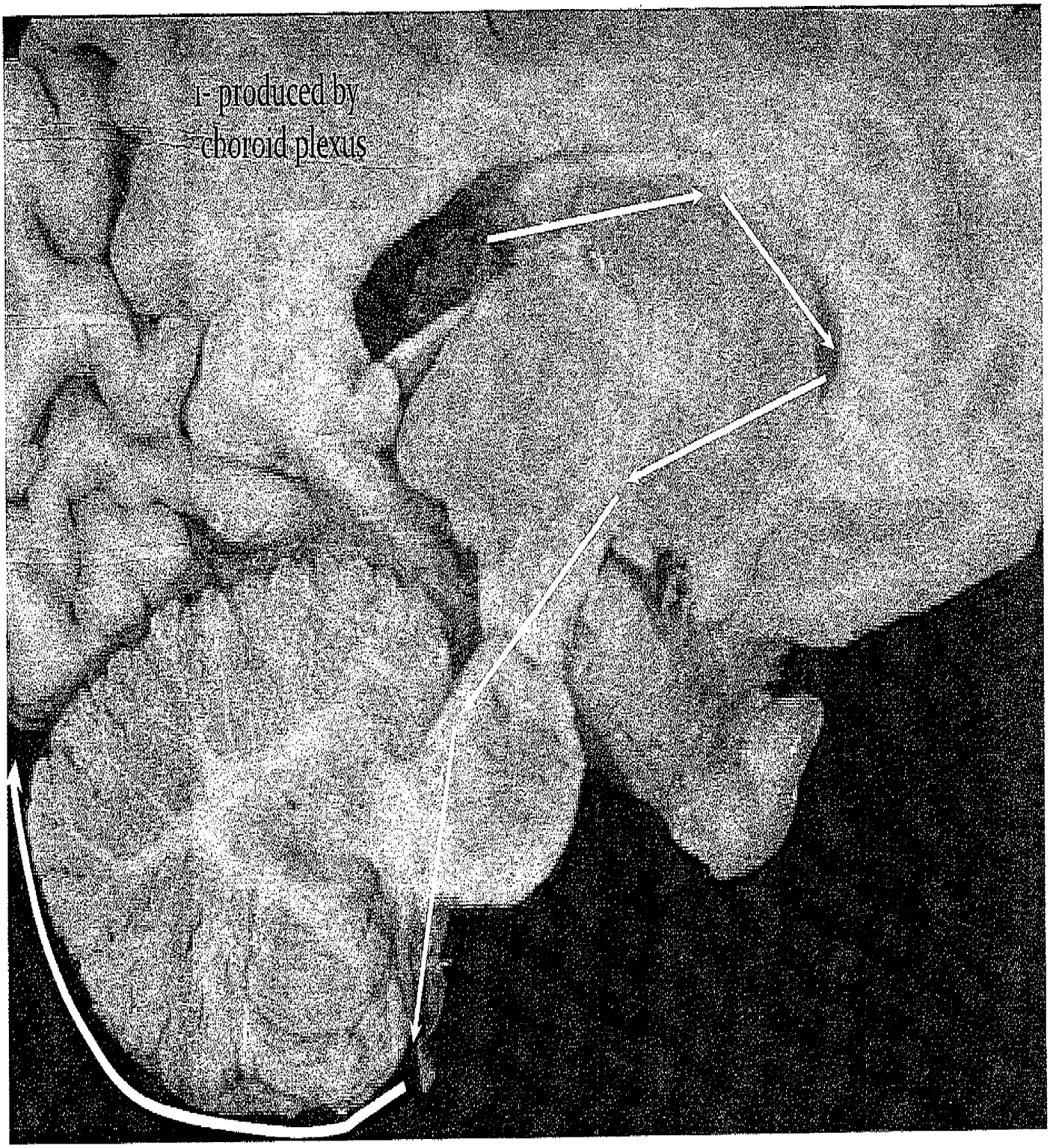

FIGURE 10: Parenteral (Intranasal) Administration of PGS (control) compared to TGF-α, following injury

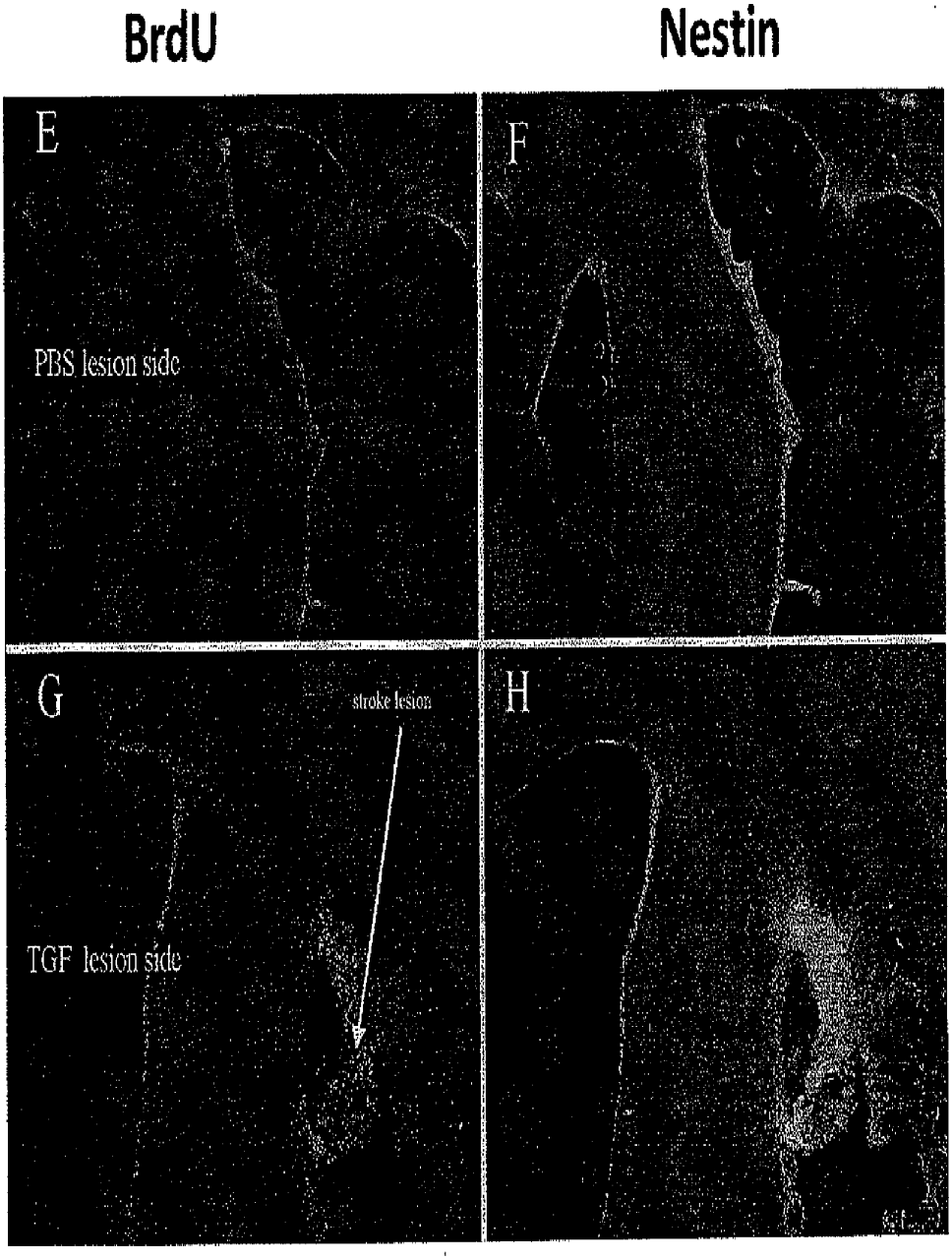

FIGURE 11: Parenteral Administration of PGS (control) compared to TGF-α in Absence of Injury

BrdU **Nestin**

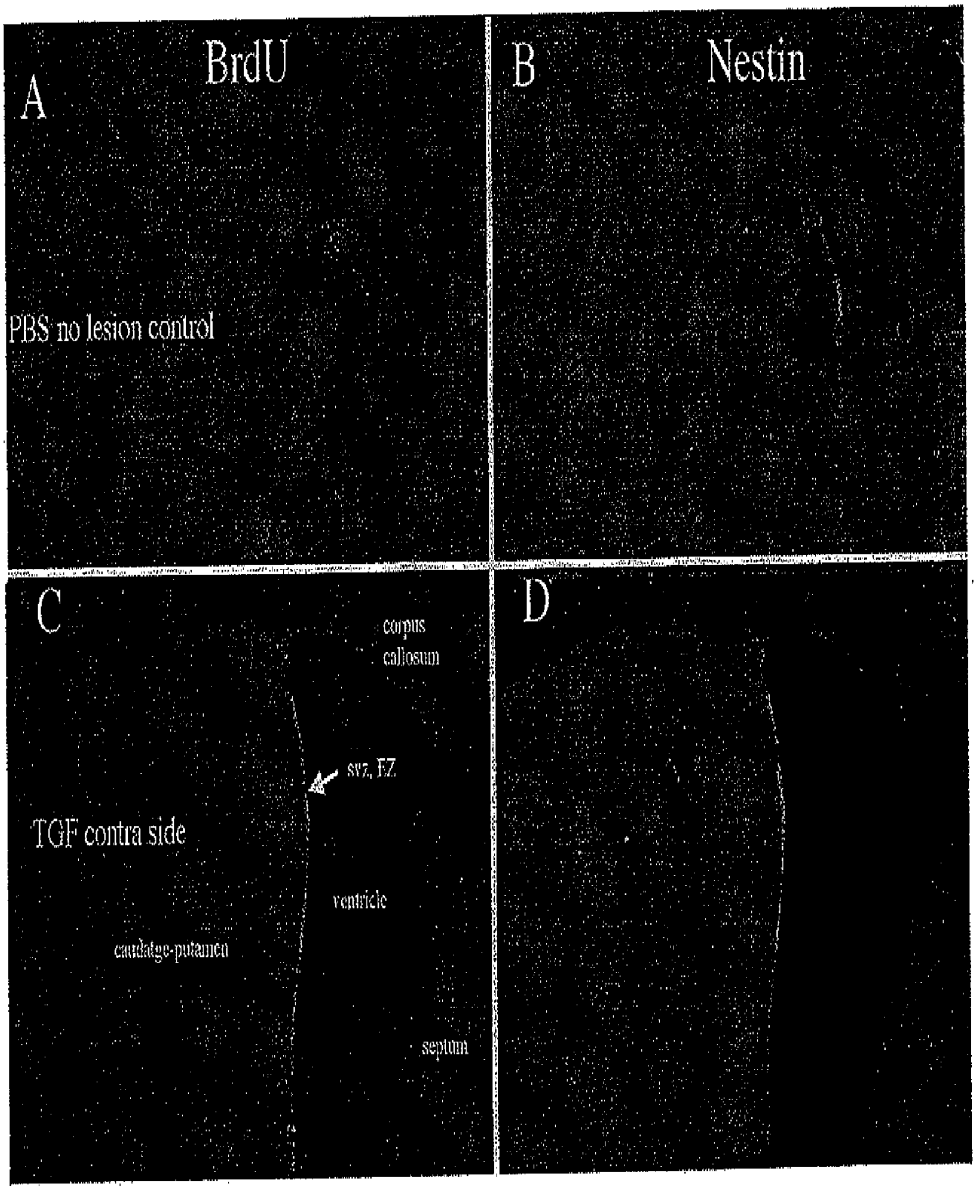

FIGURE 12: RECOVERY OF MOTOR FUNCTION